University of Alberta

Regulation of Neuronal Apoptosis in Physiological and Pathological Conditions

by

Mee-Sook Song ©

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ABSTRACT

Apoptosis is essential during development of the nervous system. However, inappropriate activation of apoptosis may contribute to neuronal loss during neurodegeneration. Therefore, it is crucial to understand the regulation of apoptosis in physiological and pathological conditions.

As a paradigm of physiological apoptosis I used sympathetic neurons deprived of nerve growth factor (NGF). Apoptosis activated by NGF withdrawal can be prevented by several mechanisms. I investigated the role of the sphingolipid ceramide. I found that the ceramide analogue, C_6-ceramide, as well as endogenous long-chain ceramides support survival of NGF-deprived sympathetic neurons. C_6-ceramide induces activation of the NGF receptor TrkA and selective activation of the PI3-kinase/Akt pathway but not the MAPK/ERK pathway. Furthermore, addition of C_6-ceramide exclusively to distal axons is sufficient to inhibit nuclear apoptosis. In several cell types, activation of the neurotrophin receptor $p75^{NTR}$ leads to ceramide accumulation. However, I demonstrate that, in sympathetic neurons, $p75^{NTR}$ activation does not generate sufficient ceramide to block apoptosis.

A second aspect of my studies aims to understand the mechanisms involved in neuronal apoptosis and axonal degeneration induced by β-amyloid peptide (Aβ), a pivotal player in Alzheimer's disease. Degeneration of basal forebrain cholinergic neurons correlates well with the degree of dementia. Basal forebrain neurons extend their axons to areas of the brain in which accumulation of Aβ is exacerbated. Hence, axons of basal forebrain neurons are preferentially exposed to insults linked to neuronal death in

Alzheimer's disease. To mimic this situation *in vitro* I cultured neurons in compartmented dishes, which allow the treatment of axons independently of cell bodies. I demonstrate that $A\beta_{1-42}$ causes rapid axonal degeneration, which leads to nuclear apoptosis. Aβ-induced axonal degeneration does not involve activation of caspases in axons, but caspases are activated in cell bodies in response to exposure of distal axons to Aβ. The calpain inhibitor calpastatin provided to distal axons protects from Aβ-induced axonal degeneration and consequently prevents apoptosis. I also demonstrate that Aβ-induced nuclear apoptosis is indistinguishable of apoptosis due to NGF deprivation. Therefore, NGF prevents apoptosis when given to cell bodies of neurons treated with Aβ in distal axons. This result supports the notion that impairment of retrograde transport of NGF or NGF signaling is involved in the death program activated by Aβ.

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LIST OF ABBREVIATIONS

Aβ: β-amyloid peptide

APP: amyloid precursor protein

BDNF: Brain Derived Neurotrophic Factor

ChAT: Choline Acetyltransferase

CNS: Central Nerve System

ERK1/2: extracellular signal-regulated kinases 1/2

FB_1: Fumonisin B_1

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

NGF: Nerve Growth Factor

PDK-1: 3-phosphoinositide-dependent kinase-1

PI3-kinase: phosphatidylinositol-3 kinases

PNS: Peripheral Nervous System

PPMP: DL-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol

SM: sphingomyelin

SMase: sphingomyelinases

Trk: Tyrosine Kinases Neurotrophin Receptor

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CHAPTER 1

BACKGROUND

1.1. General Introduction

Apoptosis is a highly regulated cellular process crucial for life. Extensive neuronal apoptosis occurs during development, particularly during target innervation, when many neuronal populations are acutely dependent on target-derived trophic factors. Apoptotic cell death is also observed in pathological conditions such as neurodegenerative disorders, stroke, spinal cord injury and AIDS-associated dementia. Therefore, understanding how apoptosis occurs and is regulated in physiological and pathological processes is crucial for developing rational therapies that may prevent neuronal death after injury or during disease.

In the first study of this thesis I use sympathetic neurons deprived of nerve growth factor (NGF) as a model for physiological apoptosis and examine the inhibition of apoptosis by the sphingolipid ceramide. Lack of NGF does not cause neurodegenerative diseases but NGF or other molecules with NGF-like activity can be used as therapeutic agents in the treatment of neurological disorders. Sympathetic neurons depend on NGF for survival, and undergo apoptosis upon NGF withdrawal both *in vivo* and *in vitro*. The molecular events that take place during apoptosis in this paradigm have been extensively characterized. Evidence from previous work suggested that ceramide protected neurons against apoptosis caused by NGF withdrawal. However, the molecular mechanisms involved in the protection of apoptosis have not been identified. The work presented here provides important information useful for the development of new alternatives to achieve neuronal survival in the absence of neurotrophins.

In order to study apoptosis in a disease-related paradigm, I focus on neuronal death induced by amyloid peptide (Aβ), which constitutes a hallmark of Alzheimer's disease. Inappropriate activation of neuronal apoptosis has been implicated in the development of neurodegenerative diseases. Axonal degeneration also contributes to the pathogenesis of several neurological disorders. The study presented here is directed to identify the mechanisms involved in the death of neurons such as basal forebrain cholinergic neurons, which are exposed to Aβ preferentially at the axons terminals. I aim to evaluate the contribution of axonal degeneration to the process of neuronal apoptosis.

Overall, the data presented in this thesis give important information for the understanding of neuronal apoptosis and axonal degeneration at the molecular level. They also provide the basis for the discovery of selective targets with therapeutic potential and the development of novel strategies to support neuronal survival.

1.2. Nerve Growth Factor and Sympathetic Neurons

1.2.1. NGF

Nerve growth factor (NGF) is the prototypic member of the neurotrophin family of growth factors involved in the survival and differentiation of specific populations of neurons (reviewed in Lewin and Barde, 1996). Members of the neurotrophin family, which include brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin 6, share a high degree of structural homology (Lewin and Barde, 1996).

NGF is constitutively produced and secreted by target tissues innervated by sympathetic and sensory neurons of the peripheral nervous system (Levi-Montalcini and Hamburger, 1951; Levi-Montalcini et al., 1954). Therefore, NGF provides trophic support to these neurons as their axons reach the final target organs. NGF is also produced in the CNS during development and throughout adult life (reviewed in Sofroniew et al., 2001). NGF-producing cells are present in the cortical target regions of basal forebrain cholinergic neurons (Sofroniew et al., 2001). NGF is important for the development and function of striatal and basal forebrain cholinergic neurons (Fagan et al., 1997; Cuello et al., 1993; reviewed in Salehi et al., 2004; Auld et al., 2002).

The NGF gene is located on human chromosome 1 and is expressed as two major splice variants (Edwards et al., 1988). The mature, fully processed form of biologically active NGF appears to be similar in all tissues and consists of a dimer of 13-kDa polypeptide chains, each of which has three intrachain disulfide bridges (McDonald et al., 1991).

1.2.2. NGF receptors

NGF binds to two surface receptors, namely TrkA and p75^{NTR} (reviewed in Chao, 2003; Sofroniew et al., 2001). TrkA is a single transmembrane 140kD protein with tyrosine kinase activity (Kaplan et al., 1991). It belongs to the Trk family of receptors, which also includes TrkB and TrkC. Trk receptors show selectivity in ligand binding. TrkA binds NGF preferentially but also NT-3 although with much less affinity. TrkB binds BDNF and NT-4/5 and TrkC binds NT-3 (reviewed in Kaplan and Miller, 1997; Huang and Reichardt, 2003).

TrkA is highly expressed in NGF-responsive sympathetic and sensory neurons. Consequently, targeted disruption of the TrkA gene in mice produces marked abnormalities in the development of these neuronal populations (Smeyne et al., 1994). In the brain, TrkA is expressed mainly in neurons of the basal forebrain and in lower levels in neurons of striatum and hippocampus (Holtzman et al., 1992) and the cortex (Salehi et al., 1996).

The extracellular domain of TrkA required for ligand binding consists of two cysteine-rich regions flanking a leucine-rich motif in the amino-terminal portion followed by two immunoglobulin-like domains in the juxtamembrane region. The intracellular part of TrkA contains two kinase domains: one at the membrane-proximal site and the second at the carboxyl terminus (reviewed in Yano and Chao, 2000).

The second NGF receptor, $p75^{NTR}$, was the first neurotrophin receptor to be cloned and characterized as a low affinity receptor (Chao, 1994). This concept, however, has changed due to indications that the binding affinity of $p75^{NTR}$ to neurotrophins is similar to that of Trk receptors, with a K_d of approximately $1x10^{-10}$ nM (Esposito et al., 2001; Rodriguez-Tebar et al., 1992). Importantly, when $p75^{NTR}$ and Trk are coexpressed, $p75^{NTR}$ enhances the ability of Trk receptors to bind and respond to neurotrophins and sharpens the discrimination of Trk receptors for their preferred neurotrophin ligands (reviewed in Barker, 2004). On the other hand, $p75^{NTR}$ binds all neurotrophins with similar affinity in most cell types (Rodriguez-Tebar et al., 1991, 1992).

$p75^{NTR}$ was also the first identified member of the tumor necrosis factor (TNF) family of receptors that includes Fas (CD95) and the tumor necrosis factor receptors (TNF-R) (reviewed in Baker, 1998). $p75^{NTR}$ has a single membrane-spanning domain and four structurally related cysteine-rich regions in the extracellular domain (reviewed in Huang, 2003). Some members of the TNF receptor family, including $p75^{NTR}$, contain an ~80 amino acid death domain that usually acts as a protein binding module for adaptor molecules that aggregate and activate caspase-8, initiating apoptosis. However, the death domain of p75^{NTR} is structurally distinct from that of other members of the family, and the signaling properties of p75^{NTR} are equally different (reviewed in Barker, 2004). With the exception of p75^{NTR}, the receptors of this family recognize cell-surface-bound ligands. Only soluble ligands are known for p75^{NTR} (reviewed in Barrett, 2000).

In the brain, p75^{NTR} is expressed by basal forebrain cholinergic neurons and developing neurons in the cortex, the brainstem and the cerebellum (Longo et al., 1993). Cholinergic neurons of the basal forebrain express both TrkA and p75^{NTR} during development and throughout adult life (Holtzman et al., 1995). In the peripheral nervous system (PNS), p75^{NTR} is expressed in NGF responsive sympathetic neurons and sensory neurons during development and in the adult (Ruit et al., 1990; Verge et al., 1989). It is also expressed by Schwann cells during development and at a much lower level in the adult (Heumann et al., 1987).

1.2.3. NGF signaling

1.2.3.1 NGF signaling through TrkA

Most of the biological effects of NGF are mediated by TrkA activation, and by the activation of multiple second messenger signaling cascades downstream of TrkA (reviewed in Huang and Reichardt, 2003; Sofroniew et al., 2001) (Fig.1-1). Binding of NGF to TrkA leads to dimerization and auto/transphosphorylation of the receptor at tyrosine residues of the activation loop. This event leads to activation of TrkA kinase activity, followed by autophosphorylation of tyrosines residues outside the activation loop (Cunningham et al., 1997). Tyrosine 490 and tyrosine 785 represent the major autophosphorylation target sites in TrkA (Loeb et al., 1994; Stephens et al., 1994). Phosphotyrosines (pTyr) serve as binding sites for specific signaling and/or adaptor proteins such as phospholipase C-γ (PLCγ) and Shc (Obermeier et al., 1994; Basu et al., 1994). Subsequent phosphorylation of recruited accessory proteins leads to the activation of cascade signaling pathways (reviewed in Huang and Reichardt, 2003; Sofroniew et al., 2001). Mutagenesis studies have shown that tyrosine residue Y490 is the principal phosphorylation site leading to the activation of the Ras/ERK and the PI3-kinase/Akt signaling pathways which mediate the growth promoting and survival effects of NGF (Stephens et al., 1994; Hallberg et al., 1998).

a. The Ras/ERK pathway

The scaffolding protein Shc is recruited to the NPXpY motif at Y490 of TrkA after NGF binding (Obermeier et al., 1993), and in turn, Shc recruits to the membrane the adaptor protein Grb2, and the guanine nucleotide exchange factor Sos. Sos activity leads to the activation of Ras by converting it from its GDP to its GTP-bound state (Obermeier et al., 1994; Stephens et al., 1994). Ras-GTP promotes the activation of the serine/threonine kinases c-Raf and B-Raf, which phosphorylate and activate MEK1 and MEK2 respectively. MEK1 and MEK2 activate in turn ERK1 and ERK2 by phosphorylation at the key residues, threonine and tyrosine (reviewed in Sofroniew et al., 2001). Extracellular Regulated Kinases 1 and 2 (ERK1/2) are members of the MAP kinase family of serine/threonine kinases that were identified by their ability to phosphorylate the microtubule-associated protein MAP2 (Cobb et al., 1991). Later, they were renamed as mitogen-activated protein kinases due to its critical role in the proliferative effects of growth factors (Rossomando et al., 1991). Activated ERK1/2 are involved in diverse neuronal functions, particularly differentiation and axonal outgrowth (Cowley et al., 1994; Pang et al., 1995). Complete inhibition of ERK activation either by pharmacological inhibition of MEK or transfection with a dominant-interfering MEK mutant, blocks NGF-induced neurite outgrowth (Cowley et al., 1994; Pang et al., 1995). The persistent ERK activation that follows NGF stimulation may induce expression of immediate early gene proteins that interact with the cyclic AMP response element binding (CREB) protein, which is critical for the survival of NGF-dependent sympathetic neurons (Riccio et al., 1999; Lonze et al., 2002). In contrast with this notion, several studies have reported that inhibition of the Ras/ERK pathway has little or no effect on NGF-dependent survival of sympathetic neurons (Virdee et al., 1995, 1996). Important for our purposes, Howe et al. (2001) have reported that NGF-induced activation of

ERK1/2 and NGF-mediated local axon growth requires internalization of NGF/TrkA complexes.

b. The PI3-kinase/Akt pathway

Membrane associated Ras-GTP binds directly to and activates PI3-kinase, thus inhibition of Ras should suppress NGF-mediated PI3-kinase activity (Rodriguez-Viciana et al., 1994; Mazzoni et al., 1999). However, PI3-kinase can also be activated in a Ras-independent manner, through Gab1 (Holgado-Madruga et al., 1997). Importantly, activation of PI3-kinase alone maintains survival of sympathetic neurons in the absence of ERK activation (Ashcroft et al., 1999).

Phosphatidylinositol-3, 4, 5-triphosphate (PIP3) generated by PI3-kinase activity induces activation of the serine/threonine kinase Akt (also known as protein kinase B (PKB)) by two known mechanisms: PIP3 binds to and activates Akt directly (Stokoe et al., 1997) and PIP3 activates the serine/threonine kinase 3-phosphoinositide-dependent kinase-1 (PDK-1) which in turn binds and activates Akt (Vanhaesebroeck et al., 2000). Available evidence indicates that Akt is critical for NGF-induced survival of sympathetic neurons (Crowder et al., 1998; Philpott et al., 1997; Virdee et al., 1999). Indeed, overexpression of constitutively active Akt maintains the survival of NGF-deprived sympathetic neuron and expression of dominant-negative Akt induces apoptosis of approximately 80% of neurons in the presence of NGF (Crowder et al., 1998; Philpott et al., 1997). Akt negatively regulates the activity of pro-apoptotic molecules, including Bad, a member of Bcl-2 family (Datta et al., 1997), members of the Forkhead family of transcription factors that mediate transcription of pro-apoptotic genes (Brunet et al., 1999; Kops et al., 1999) and glycogen synthase kinase-3 (Pap et al., 1998).

c. Other NGF/pTrkA activated pathways

In addition to the two pathways described above, TrkA phosphorylation leads to the recruitment and activation of several additional and important signaling pathways that involve the activation of phospholipase C-γ (PLC-γ), protein kinase C (PKC), FRS/SNT, AbL as well as rAPS- and SH_2-B mediated pathways (reviewed in Sofroniew et al., 2001; Huang and Reichardt, 2003). Analysis of these pathways escapes the focus and purpose of this thesis.

1.2.3.2 NGF signaling through p75 ^{NTR}

Activation of certain receptors of the TNF family, including p75^{NTR}, induces cell death (Barrett, 2000). Evidence from *in vitro* and *in vivo* studies suggests a role for p75^{NTR} in apoptosis, although the precise mechanism underlying this process remains unclear (Roux et al., 2002). p75^{NTR} mediates developmentally programmed death in the embryonic chick retina (Bredesen et al., 1997; Carter et al., 1997). In sympathetic neurons, BDNF signaling via p75^{NTR} induces apoptosis *in vitro* and sympathetic neuronal death is delayed in p75^{NTR} homozygous null mice (Bamji et al., 1998).

Some of p75^{NTR} effects seem to be mediated by accumulation of the sphingolipid ceramide. Binding of neurotrophins (including NGF) to the p75^{NTR} leads to the activation of sphingomyelin (SM) hydrolysis and ceramide accumulation in NIH-3T3 cells and T9 cells (Dobrowsky et al., 1994, 1995). p75^{NTR}-dependent ceramide production was also demonstrated in other several systems (Brann et al., 1999, Brann et al., 2002, Casaccia-Bonnefil et al., 1996; DeFreitas et al., 2001). Therefore ceramide is often considered one of the downstream effectors of p75^{NTR} (Barrett, 2000; Mamidipudi et al., 2002).

Signaling pathways activated downstream of p75 ^{NTR} are slowly emerging and became the focus of intensive recent research (reviewed in Miller and Kaplan, 1998; Sofroniew et al., 2001).

1.2.4. NGF Deprivation of Sympathetic Neurons

Sympathetic neurons depend on NGF for survival *in vivo* and *in vitro* (Levi-Montalcini and Angeletti, 1968). Apoptosis of sympathetic neurons deprived of NGF *in vitro* has been studied extensively and provides an excellent model of mammalian programmed cell death. One advantage of this *in vitro* model is that sympathetic neurons represent a homogeneous population of cells that undergo complete death in a synchronous and reproducible manner (reviewed in Deshmukh and Johnson, 1997). NGF withdrawal triggers a cell death program characterized by morphologic, biochemical and genetic features of classic apoptosis (see page 24).

NGF withdrawal causes the rapid dephosphorylation of TrkA leading to a cascade of well-characterized events. The most prominent early events include alterations in signaling pathways, such as decrease in PI3-kinase and ERK activities (Deckwerth et al., 1993). Increased production of reactive oxygen species also occurs at an early time after NGF deprivation and is followed by increased c-jun activation (Greenlund et al., 1995). Subsequently, Bax is translocated to the mitochondria where it leads to the release of cytochrome c (Deshmukh and Johnson, 1998). As a result caspases are activated (Deshmukh and Johnson, 1996) (Fig 1-2). Importantly, apoptosis of sympathetic neurons requires protein synthesis and therefore is prevented by the protein synthesis inhibitor cycloheximide (Martin et al., 1992).

Many of the early consequences of NGF deprivation in sympathetic neurons are reversible upon the addition of growth factor (reviewed in Chang et al., 2002). Eventually however, neurons reach a point of no-return in the cell death program known as commitment-to-die points. Two points of commitment-to-die have been recognized. The first one takes place upon cytochrome c release and before caspase activation. Caspase inhibition can still rescue neurons even after cytochrome c release. The commitment-to-die point 1 can be defined as the latest time at which NGF re-addition can rescue NGF-deprived neurons. The second commitment-to-die point 2 is defined by the loss of mitochondrial membrane potential. Re-addition of NGF after the "commitment-to-die point 2" can no longer rescue neurons from apoptosis (reviewed in Chang et al., 2002; Chang and Johnson, 2002).

1.2.5. NGF and Neurodegenerative Disorders: Alzheimer's disease

NGF is important in the survival and maintenance of adult basal forebrain cholinergic neurons (Hefti et al., 1987). Importantly, the degree of dementia in Alzheimer's disease correlates with the degeneration of basal forebrain cholinergic neurons (Perry et al., 1978; Whitehouse et al., 1982). NGF is synthesized in the cortex and the hippocampus, and is transported retrogradely to cell bodies of basal forebrain cholinergic neurons (reviewed in Yuen et al., 1996; Rattray et al., 2001). Studies *in vivo* have clearly shown that endogenous NGF contributes to the development, maintenance and function of basal forebrain cholinergic neurons with profound influence on choline acetyltransferase (ChAT) activity, acetylcholine release and cell body size (Vantini et al., 1989; Fagan et al., 1997; Debeir et al., 1999). Moreover, NGF acting on basal forebrain cholinergic neurons seems critical for certain learning and memory functions (Gutierrez et al., 1997; Woolf et al., 2001).

Due to the significance of the cholinergic deficit in Alzheimer's disease brain and the relationship of NGF with basal forebrain cholinergic neurons, it is reasonable that alterations in NGF and its receptor TrkA have been observed in Alzheimer's disease brain. The levels of NGF in Alzheimer's disease brain are normal or increased in target regions of basal forebrain cholinergic neurons, including the hippocampus and the parietal cortex (Crutcher et al., 1993; Scott et al., 1995; Fahnestock et al., 1996; Siegal et al., 2000). On the other hand, NGF levels in nuclei of the basal forebrain are reduced (Scott et al., 1995). Concomitantly, decreased expression and immunoreactivity of TrkA has been observed in basal forebrain cholinergic neurons in Alzheimer's disease brain (Mufson et al., 1997; Salehi et al, 2004). This reduction likely contributes to the cholinergic deficits present in Alzheimer's disease by impairing the normal maintenance of cholinergic neurons by NGF (Salehi et al., 2004). These observations support the notion that in Alzheimer's disease there is an impairment of NGF retrograde transport or signaling from target tissues of basal forebrain cholinergic neurons (Salehi et al., 2004). In fact, failed retrograde transport of NGF has been proven in animal model of Down Syndrome, Ts65Dn mice (Salehi et al., 2004). Importantly, all patients with Down Syndrome older than 40 years show the pathological hallmarks of Alzheimer's disease and a number of them develop dementia in later life. Moreover Down Syndrome is characterized by severe degeneration of basal forebrain cholinergic neurons and cholinergic deficiency. All these features of Down Syndrome seem to result from the overexpression of normal genes located on chromosome 21 which includes the gene for amyloid precursor protein (APP). Therefore, Ts65Dn mice are useful for studying degeneration of basal forebrain cholinergic neurons and other aspects of Alzheimer's disease such as impairment of NGF signaling (Salehi et al., 2004).

In addition, failure of axonal transport has been demonstrated in human cortical cells from postmortem Alzheimer's disease brain (Dai et al., 2002). Moreover, animal studies indicate that cholinergic lesions leading to cognitive disturbances can be decreased by NGF treatment (Fischer et al., 1987; Hefti et al., 1989). In particular, intraparenchymal delivery of NGF to the adult primate brain by gene transfer can prevent the degeneration of basal forebrain cholinergic neurons (Tuszynski et al., 1996).

1.3. Ceramide

Over the last two decades extensive research has demonstrated the role of sphingolipids and their metabolites as important cellular signaling mediators. Sphingolipids function as effector molecules and have important roles in stimulus-mediated signaling and in the regulation of diverse cellular processes. In particular ceramide, the central molecule in sphingolipid metabolism, mediates cellular functions ranging from proliferation and differentiation to growth arrest and apoptosis (reviewed in Hannun and Luberto, 2000; Pfeilschifter and Huwiler, 2000; van Blitterswijk et al., 2003).

Ceramide was first isolated from human brain. The name ceramide was coined by Thudichum in 1884 and refers to a family of naturally occurring N-acylated sphingosines.

A variety of physiological stimuli, such as cytokines and growth factors, induce changes in ceramide levels (reviewed in Mathias, 1998). Ceramide is also generated in response to stress stimuli such as ionizing and UV radiation, chemotherapeutic drugs, serum withdrawal, or oxidative stress (Mathias et al., 1998).

1.3.1. Ceramide Structure

Ceramide consists of a sphingoid backbone of sphingosine (D-erythro-1, 3-dihydroxy-2-amino-4-octadecene), linked to fatty acids of variable length (2-28 carbon atoms) (Fig. 1-3). The most abundant isomer of natural ceramide is the D-erythro-C_{18}-ceramide, which also has the greatest bioactivity (reviewed in Kolesnick, 2000).

Due to its extreme hydrophobicity ceramide is present exclusively in cellular membranes and does not exist as cytosolic or as free ceramide (Kolesnick et al., 2000). Consequently, targets of ceramide require that they are either transiently or permanently associated with cellular membranes (Venkataraman and Futerman, 2000).

1.3.2. Ceramide Synthesis and Metabolism

1.3.2.1. The *de novo* synthesis of ceramide

Cellular ceramide is generated by the *de novo* synthesis pathway and from hydrolysis of sphingomyelin. The initial step of the *de novo* synthesis of ceramide involves the condensation of L-serine with palmitoyl-CoA in a reaction catalyzed by the enzyme serine palmitoyltransferase (Fig.1-4). This process occurs at the cytosolic side of the endoplasmic reticulum (Mandon et al., 1992). The action of serine palmitoyltransferase yields 3-ketosphinganine, which is rapidly reduced to sphinganine by the enzyme NADPH-dependent 3-keto-dihydrosphinganine reductase (Mandon et al., 1992). The amino group of sphinganine is acylated by sphinganine N-acyltransferase (also referred as ceramide synthase) to form dihydroceramide (Merrill and Wong, 1986). The high activity of ceramide synthase does not allow accumulation of free sphingoid base, which can be cytotoxic (Merrill and Wong, 1986). Important for the work presented here, ceramide synthase is inhibited by the fungal toxin Fumonisin B1, which is structurally similar to sphinganine (Wang et al., 1991). Dihydroceramide is converted to ceramide by the insertion of a trans-double bond at the 4, 5 position of the sphinganine backbone. This reaction is catalyzed by dihydroceramide desaturase (Rother et al., 1992; Merrill and Wong, 1986). The double bond is the only structural variation between dihydroceramide and ceramide, but accounts for a significant difference in biological activity, as D-*erythro*-dihydroceramide is, in most cases, biologically inactive (Bielawska et al., 1993).

1.3.2.2. Generation of ceramide by sphingomyelin hydrolysis

A very important pool of bioactive ceramide is also generated from the hydrolysis of sphingomyelin (Fig.1-4). This pathway is often referred as the sphingomyelin cycle (Hannun, 1994). The enzymes that catalyze this reaction are sphingomyelinases which are phospholipase C isoforms directed toward sphingomyelin. There are at least seven classes of sphingomyelinases distinguished by their optimum pH of action, cation requirement, and cellular location (reviewed in Levade and Jaffrezou, 1999; Hannun et al., 2001). The products of sphingomyelinases action are ceramide and phosphocholine. Activation of SMases in response to extracellular signals was considered as the main pathway for generation of bioactive ceramide. However, current evidence supports the idea that both pathways of ceramide generation (sphingomyelin hydrolysis and *de novo* synthesis) are equally important in ceramide signaling (Pettus et al., 2002).

1.3.2.3. Ceramide Metabolism

Ceramide is an intermediate in sphingolipid synthesis. It is rapidly converted to sphingomyelin and glycosphingolipids which have important cell biological functions (Luberto and Hannun, 1999) (Fig.1-4, 1-5). Conversion of ceramide to sphingomyelin takes place at the luminal face of the Golgi apparatus (Lipsky and Pagano, 1985). On the other hand, synthesis of glycosphingolipids from ceramide occurs at the cytosolic face of the Golgi and in pre-Golgi compartments (Futerman and Pagano, 1991). The latter process involves conversion of ceramide to glucosylceramide by transfer of glucose from UDP-glucose in a reaction catalyzed by glucosylceramide synthase. Inhibition of glucosylceramide synthase leads to accumulation of *de novo* synthesized ceramide (de Chaves et al., 1997), which has been used as an strategy to elevate endogenous ceramide levels (see below). Sphingomyelin and glycosphingolipids are transported by vesicular transport to specific lipid microdomains (lipid rafts and caveolae) on the cell surface. Most of sphingomyelin is confined to the outer leaflet of plasma membrane (reviewed in Testi, 1996).

Ceramide is also the substrate of ceramidases that yields sphingosine, which in turn may be phosphorylated to sphingosine 1-phosphate. Sphingosine 1-phosphate is a potent signaling mediator (Spiegel and Milstien, 2003). In addition phosphorylation of ceramide by ceramide kinase generates ceramide-1 phosphate (Hannun and Luberto, 2000), which also serves as a cellular mediator (Gomez-Munoz, 2004).

1.3.3. Ceramide as a Second Messenger

Ceramide generation occurs in diverse cellular processes including differentiation, proliferation, senescence, cell cycle arrest, survival and apoptosis. Ceramide was first implicated as a signaling intermediate in differentiation of HL-60 cells (Okazaki et al., 1989). This pioneering study demonstrated that treatment of HL-60 cells with 1α, 25-dihydroxyvitamin D activates a neutral sphingomyelinase leading to accumulation of ceramide and causing cell differentiation.

It was later recognized that ceramide accumulates and mediates apoptosis in non-neuronal cells in response to a variety of stress stimuli such as TNF-α, Fas ligand, ionizing radiation, heat shock, oxidative stress and growth factor withdrawal (van Blitterswijk et al., 2003). The finding that ceramide activates the protease prICE, inducing poly (ADP-ribose) polymerase (PARP) cleavage (Smyth et al., 1996) and that apoptosis induced by ceramide is blocked by Bcl-2 (Zhang et al., 1996) demonstrated the direct involvement of ceramide with regulators of apoptosis.

Although ceramide is involved in several diverse cellular processes, only a few target proteins for ceramide have been identified, namely protein kinases PKCζ and Raf-1, kinase suppressor of Ras (KSR), cathepsin D, ceramide-activated protein phosphatases PP1 and PP2A (van Blitterswijk et al., 2003; Hannun and Luberto, 2000; Hannun, 1996). Work from our laboratory indicated that, in sympathetic neurons, ceramide activates protein phosphatase 1 and causes dephosphorylation of the retinoblastoma gene product pRb (Plummer et al., 2005).

Lipid second messengers usually bind with high affinity to a well-defined consensus motif in the primary sequence of their target proteins. Such motifs have been defined for phosphoinositides and diacylglycerol, but not for ceramide (van Blitterswijk et al., 2003). Based on the structural similarity between diacylglycerol and ceramide, it has been suggested that ceramide might bind to the cysteine-rich domains of PKCζ, Raf-1 and KSR. However, this still remains to be definitively proven. There is some evidence for direct binding of natural ceramides to PKCζ (Huwiler et al., 1998; Gopee et al., 2003) and cathepsin D (Heinrich et al., 1999).

1.3.4. Ceramide Signaling in Neurons

One of the first indications that ceramide was important for signaling in neurons was presented by Harel and Futerman (1993). They showed that inhibition of sphingolipid synthesis by Fumonisin B1 prevents axon outgrowth in hippocampal neurons and that the effect is reversed by treatment with C_6-ceramide. Accordingly, work from the same laboratory demonstrated that inhibition of the conversion of ceramide to glucosylceramide causes an increase in axonal branching in hippocampal neurons (Schwarz et al., 1995). Additional evidence indicated that ceramide promotes differentiation of neuroblastoma cells, cerebellar granule neurons (Riboni et al., 1995; Tettamanti et al., 1996) and dendrite formation in cerebellar Purkinje cells (Furuya et al., 1998).

The role of ceramide as a regulator of neuronal apoptosis is complex since ceramide induces or protects from apoptosis depending on the neuronal type. C_2-ceramide protects hippocampal neurons from apoptosis induced by excitotoxic stimuli including β-amyloid (Goodman et al., 1996; Mattson et al., 1997). In hippocampal neurons, ceramide stimulates axonal outgrowth immediately after neuronal plating (Brann et al., 1999) and induces apoptosis at later stages (Brann et al, 2002). Ceramide also induces apoptosis of cortical (Willaime et al., 2001), mesencephalic (Brugg, 1996) and sensory neurons (Ping et al., 1998). In motoneurons, ceramide induces survival or death according to the concentration used (Irie et al., 1998).

In sympathetic neurons, ceramide inhibits axonal growth in the presence of NGF (de Chaves et al., 1997, 1998), and delays apoptosis induced by NGF deprivation (Ito et al., 1996; Nair et al., 2000; de Chaves et al., 2001). Nair et al. (2000) reported that the protective effect of ceramide in apoptosis of sympathetic neurons deprived of NGF might be mediated by antagonizing oxidative stress and c-jun induction. However, the mechanisms underlying the protection by ceramide in apoptosis induced by NGF withdrawal remain unclear.

The first study of this thesis aims to characterize the anti-apoptotic effect of ceramide in NGF-deprived sympathetic neurons and to investigate the mechanisms involved in ceramide-induced neuronal survival.

1.3.5. Tools to Study Ceramide Action

Two main strategies have been developed to study the cellular actions of ceramide; first to treat cells with exogenous ceramides and second to stimulate the accumulation of endogenous ceramides.

Long-chain ceramides are insoluble in aqueous solutions and therefore cannot be easily delivered to cells. Instead, short-chain analogues of ceramide containing a shorter fatty acid moiety (i.e., C_2-ceramide (acetyl ceramide) and C_6-ceramide (hexanoyl ceramide)) have been used extensively to study the cellular effects of ceramide (Hannun and Luberto, 2000; Ghidoni et al., 1999). Short-chain ceramides are still hydrophobic but once dissolved in organic solvents, they swell in aqueous solutions forming micelles. Short-chain ceramides rapidly partition into membranes (Simon et al., 1998) and are likely endocytosed into endosomes and targeted to the Golgi (Babia et al., 1994).

The use of short-chain ceramides has been criticized in the past because they might have different properties with respect to ordering/packing effect on the phospholipids membranes (Ghidoni et al., 1999). The use of short-chain ceramides however, has been validated by several discoveries. First, C_2-ceramide occurs in mammalian cells (Abe et al., 1996; Lee et al., 1996). Second, short-chain ceramides mimic many of the biological actions that take place in response to agonists that generate long-chain ceramides by activation of the sphingomyelin cycle (Hannun and Luberto, 2000). Third, short-chain ceramides given exogenously to cultured cells are converted to long-chain ceramides (Ogretmen et al., 2002). Thus, some of the effects observed with short analogues might in fact be due to long-chain ceramides. In addition, the biologically inactive C_2- and C_6-dihydroceramide provide excellent negative controls for studies using short chain ceramides.

An alternative approach to achieve elevated levels of cellular ceramide is to manipulate the *de novo* pathway of sphingolipid synthesis by using inhibitors of ceramide metabolism. The use of DL-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) (which inhibits glucosylceramide synthesis) has been established as a valuable tool (de Chaves et al., 1997) (Fig.1-5). The efficiency of *threo*-PPMP but not *erythro*-PPMP in inhibiting the conversion of ceramide into complex glycosphingolipids in sympathetic neurons has been well demonstrated (de Chaves et al., 1997). Importantly, it has been shown in sympathetic neurons that the effect of short chain ceramide mimic the effect of natural ceramides accumulated by inhibition of conversion into glucosylceramide (de Chaves et al., 1997).

1.4. Neurodegeneration in Alzheimer's disease

1.4.1. Pathophysiology of Alzheimer's disease

Alzheimer's disease is a progressive neurodegenerative disorder manifested by impairment of memory and cognition. The risk of Alzheimer's disease increases dramatically over the age of 70 and it has been predicted that the incidence of Alzheimer's disease will increase three fold worldwide in the next 50 years (reviewed in Mattson, 2004).

Neurological hallmarks of Alzheimer's disease include the presence of neuritic plaques and neurofibrillary tangles, synaptic loss and/or dysfunction, decreased neuronal metabolism (indicated by mRNA level, glucose metabolism and cytochrome oxidase) and the loss of multiple neurotransmitter systems particularly cholinergic neurons (reviewed in Salehi et al., 2003, 2004). Neuritic plaques are composed of extracellular fibrils of β-amyloid peptide (Aβ) surrounded by dystrophic neurites, activated microglia and astrocytes (reviewed in Selkoe 2004; Hardy and Selkoe, 2002). Neurofibrillary tangles are intracellular accumulations of paired helical filaments of hyperphosphorylated tau, a microtubule-associated protein. Neuritic plaques and neurofibrillary tangles are found in selective regions of the brain, in particular in areas involved in memory, behavior and cognition, such as hippocampus, entorhinal cortex and neocortex (reviewed in Selkoe, 2004).

Accumulation of Aβ in neuritic plaques has been proposed as the primary event in Alzheimer's disease pathogenesis (reviewed in Selkoe, 2004; Hardy and Selkoe, 2002). Although some evidence suggests that the degree of dementia correlates with neuritic plaque formation (Caramelli et al., 1998; Cummings et al., 1995), most evidence argues against this correlation (Terry et al., 1991; Dickson et al., 1995; reviewed in Parihar 2004). Instead, a much better correlation exists between the degree of dementia and the degree of degeneration of cholinergic neurons from the basal forebrain (Perry et al., 1978; Whitehouse et al., 1982; Biere et al., 1995). In addition to Aβ in senile plaques, Alzheimer's disease brains also contain soluble assemblies of Aβ. An emerging view supports the notion that soluble Aβ rather than insoluble deposits of Aβ in plaques are toxic and responsible of the cognitive deficits characteristic of Alzheimer's disease (Cleary et al., 2005; reviewed in Adlard and Cummings, 2004).

The extent to which Alzheimer's disease is explained by genetic factors is uncertain. Mutations of three genes for amyloid precursor proteins (APP) on chromosome 21, presenilin 1(PS1) on chromosome 14 and presenilin 2 (PS2) on chromosome 1 have been linked to the familial form of Alzheimer's disease (reviewed in Ashford and Mortimer, 2002; Tanzi and Bertram., 2001). However, familial Alzheimer's disease accounts for only small proportion of Alzheimer's disease patients, generally with onset at an early age. The majority of Alzheimer's disease cases (~95%) is non-familial or sporadic, and present with late onset. The only risk factor that has been consistently found associated with the late onset forms of Alzheimer's disease is the ε4 allele of the *APOE* gene on chromosome 19 (reviewed in Ashford and Mortimer 2002; Tanzi and Bertram, 2001). Recently, additional genetic factors have been linked to some cases of late-onset Alzheimer's disease (reviewed in Tanzi and Bertram, 2001; Ashford and Mortimer, 2002). Both forms of Alzheimer's disease are almost indistinguishable phenotypically.

1.4.1.1. β-Amyloid Peptide (Aβ) Hypothesis

Aβ, the major component of neuritic plaques, is a 39-43 amino acid peptide derived from APP by sequential enzymatic cleavage (Selkoe, 2004). APP is an ubiquitously expressed integral membrane protein that contains a single membrane spanning domain, a large extracellular glycosylated N-terminus and a shorter cytoplasmic C-terminus. APP is the first protein known to undergo constitutive endoproteolysis within its transmembrane domain (Selkoe, 2004). Endoproteolysis has since been identified in the cleavage of other members of the APP and Notch families (Lammich et al., 2002). The major pathway of APP cleavage involves the action of α-secretase at the N-terminus followed by γ-secretase at the C terminus. These two enzymatic activities generate APPs-α and p3, leaving the C-terminal domain of APP at the membrane (Fig 1-6).

Alternatively, APP can serve as a substrate for the β-site APP cleaving enzyme (BACE) or β-secretase which cleaves at the N-terminus generating APPs-β and a C-terminal fragment which in turn is cleaved by γ-secretase to form Aβ (reviewed in Mattson, 2004) (Fig 1-6). Aβ is produced constitutively under normal metabolic conditions and is present in normal cerebrospinal fluid and human plasma (Shoji et al., 1992; Haass et al., 1992; Seubert et al., 1992).

Depending upon the site of γ-secretase cleavage, the resulting peptides are short (40 amino acids: $A\beta_{40}$) or long (42 amino acids: $A\beta_{42}$). The majority of Aβ generated is $A\beta_{40}$ however, $A\beta_{42}$ is the major species present in plaques of Alzheimer's disease brains (Iwatsubo et al., 1994), and is likely critical in the onset and progression of Alzheimer's disease (Cummings et al., 1996; reviewed in Hardy, 1997).

According to the Aβ peptide hypothesis, accumulation of Aβ in the brain is the primary event in Alzheimer's disease. Several lines of evidence support this view: First, all patients with Alzheimer's disease accumulate deposits of $A\beta_{42}$ in brain regions important for memory and cognition (Masters et al., 1985). Second, $A\beta_{42}$ accumulation precedes all other pathological features of Alzheimer's disease (Masters et al., 1985). Third, *in vivo* and *in vitro* models of three mutated genes (amyloid precursor protein, presenilin 1 and presenilin 2) show an increase of the cellular production and extracellular accumulation of $A\beta_{42}$ (Citron et al., 1997; Cai et al., 1993; Suzuki et al., 1994). Fourth, ApoE4 polymorphism, the major genetic risk factor for late onset Alzheimer's disease is linked to altered clearance and accumulation of diffuse and fibrillar $A\beta_{42}$ (Bales et al, 1997; Corder et al., 1993). Fifth, overexpression of mutant human APP in transgenic mice (PDAPP) leads to progressive accumulation of $Aß_{42}$ and formation of Aß plaques associated with microgliosis, astrocytosis, and neuritic/synaptic dystrophy (Games et al., 1995; Irizarry et al., 1997). Sixth, $A\beta_{42}$ accumulation can lead to secondary neurofibrillary tangle formation, but the opposite sequence of events has not been demonstrated (Hardy et al., 1998; Lewis et al., 2001).

The ability of Aβ to induce neurotoxicity in cell cultures and *in vivo* was originally related to its fibrillization (Lorenzo et al., 1994; Estus et al., 1997; Ye et al., 2004; Pike et al., 1991, 1993; Kienlen-Campard and Octave, 2002). However, it was later demonstrated that soluble Aβ also induces neuronal apoptosis (Pillot et al., 1999). The level of soluble Aβ increases prior to Aβ plaque formation (Teller et al., 1996; Pillot et al., 1999; reviewed in Walsh and Selkoe, 2004) suggesting that accumulation of soluble Aβ could initiate the pathology of Alzheimer's disease or directly contribute to neurotoxicity. Oligomeric Aβ has been found *in vivo* (Gong et al., 2003), and in cultured neurons (Walsh et al., 2002). Interestingly, soluble oligomeric Aβ is more effective than fibrillar Aβ in inducing neuronal death in cultured neurons (Dahlgren et al., 2002; Stine et al., 2003; Trommer et al., 2005).

An indication that oligomeric Aβ plays an important role in Alzheimer's disease is offered by the finding that cerebral microinjection of cell-conditioned medium containing oligomers and monomers of Aβ, but not fibrils, markedly inhibited hippocampal long-term potentiation in rats *in vivo* (Walsh et al., 2002). Moreover, oligomeric Aβ is shown to induce the inhibition of long term potentiation under conditions of depletion of monomer Aβ (Walsh et al., 2002) and to impair cognitive function and learning behavior (Cleary et al., 2005).

1.4.1.2. Cholinergic Hypothesis

The cholinergic hypothesis, first proposed by Bartus et al. (1982) and Coyle et al. (1983), established that the significant dysfunction of cholinergic activity in brains of aging, and especially, demented patients plays a critical role in memory loss and related cognitive disorders. Similar memory deficits can be induced artificially by lesions that damage cholinergic input from the basal forebrain to the neocortex and hippocampus, indicating that cholinergic blockade causes memory dysfunction (reviewed in Terry and Buccafusco, 2003). In Alzheimer's disease there is consistent loss of cholinergic markers in the cortex, the hippocampus and the basal forebrain (Geula et al., 1994; Bartus et al., 2000; reviewed in Giacobini, 2003). Cholinergic neurons of the basal forebrain from which innervations to the neocortex and hippocampus originate, display severe atrophy (Whitehouse et al., 1982; Salehi et al., 1994; Emre et al., 1993) and decreased in ChAT activity (Lehericy et al., 1993; DeKosky et al., 2002; Davis et al, 1999). Based on these facts the "cholinergic hypothesis" proposes that loss of cholinergic function in the CNS is the main contributor to the dementia and cognitive decline associated with Alzheimer's disease (Geula et al., 1994; Bartus et al., 2000). Extensive evidence from studies using anti-cholinergic drugs, examining the cholinergic system in patients with impaired memory and cognition as well as data from studies that attempted to improve memory and cognition with cholinomimetic drugs support this hypothesis (reviewed in Roberson and Harrell, 1997; Auld et al., 2002) .

1.4.1.3. Link between Cholinergic and Aβ Hypotheses

The question arises as to whether, cholinergic dysfunction or Aβ accumulation is the central event in Alzheimer's disease, and which of these is responsible for the deficits in cognition and memory (reviewed in Auld et al., 2002; Roberson and Harrell, 1997). The possibility of a unified hypothesis is emerging based on evidence indicating that: i) choline uptake and ACh release are potently inhibited by Aβ in the hippocampus and cortex (Kar et al., 1996, 1998; Vaucher et al., 2001), ii) cultured cholinergic neurons from basal forebrain are vulnerable to Aβ (Jhamandas and MacTavish, 2004; Zheng et al., 2002; Pedersen et al., 1996; Hoshi et al., 1997), iii) Aβ regulates ChAT, thus altering cholinergic neurotransmission (Dobransky et al., 2003), iv) transgenic mice overexpressing mutant human amyloid precursor protein (PDAPP) show cholinergic neuropathy (German et al., 2003). Collectively, these results suggest the existence of functional interrelationships between Aβ and cholinergic neurons that are vulnerable in Alzheimer's disease pathology.

1.4.2. Neuronal Apoptosis in Alzheimer's disease

Apoptosis is a genetically regulated and evolutionarily conserved cellular process (reviewed in Zimmermann and Green, 2001). In multicellular organisms apoptosis is crucial for development, organ morphogenesis, tissue homeostasis and defense against infected or damaged cells (Zimmermann and Green, 2001). Apoptosis is characterized by plasma membrane blebbing, translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane, cytoplasm shrinking, nuclear chromatin condensation and fragmentation of DNA into oligonucleosomal units (reviewed in Vila and Przedborski, 2003). Cells dying by this mechanism are eventually engulfed by neighboring cells (e.g. phagocytes) without causing inflammation (reviewed in Lauber et al., 2004).

Apoptosis occurs by the extrinsic (death receptor) pathway and/or the intrinsic (the mitochondrial) pathway (Fig. 1-7). Both pathways of apoptosis ultimately lead to activation of a family of cysteine proteases called caspases. Caspases are pivotal mediators of mammalian apoptosis in general (Zimmermann and Green, 2001; Putcha and Johnson, 2004; Hengartner and Bryant, 2000) and play a critical role in apoptosis in neurons (reviewed in Yuan and Yankner, 2000; Troy et al., 2002; Vila and Przedborski, 2003). Over a dozen caspases have been identified in humans; about two-thirds of these functions in apoptosis (Putcha and Johnson, 2004).

The intrinsic (mitochondrial) pathway of apoptosis is activated in response to extracellular cues and/or internal insults such as DNA damage (reviewed in Hengartner and Bryant, 2000). As a consequence, anti-apoptotic (Bcl-2 and Bcl-x_L) and pro-apoptotic members of the Bcl-2 family (Bax and Bak) compete for binding to Bid at the mitochondrial membrane. If the apoptotic tendency is stronger than the anti-apoptotic, the release of mitochondrial apoptogenic factors such as cytochrome c into the cytoplasm occurs (Fig. 1-7). Once released to the cytoplasm, cytochrome *c* promotes assembly of Apaf-1 and procaspase-9 into the apoptosome, which contains active caspase-9 and is capable of proteolytically processing caspase-3.

In the extrinsic pathway, death receptors such as Fas/CD95 or tumor necrosis factor receptor 1 (TNFR1) are activated by binding of their ligands. Upon activation of the death receptors, a cascade of sequential reactions takes place, finally leading to cell death (Fig. 1-7). Caspase 8 is activated by association of the death receptors with adaptor proteins such as FADD or TRADD. Activated caspase 8 activates caspase-3 directly. Caspase-8 also activates other caspases indirectly by cleaving the cytosolic proapoptotic protein, Bid, which is a member of the Bcl-2 family. Caspase 8-mediated cleavage of Bid results in Bid translocation to the mitochondria where it promotes cytochrome c release.

The extrinsic and intrinsic pathways converge at the levels of caspase 3 activation. Cross-talk and integration between the two pathways is provided by Bid (reviewed in Hengartner, 2000). Importantly, the two apoptotic pathways also function independently of each other (Hengartner and Bryant, 2000).

Inappropriate initiation of neuronal apoptosis has been implicated in the development of neurodegenerative disorders (reviewed in Ekshyyan, 2004; Vila and Przedborski, 2003; Yuan and Yankner, 2000). In brains of patients with neurodegenerative disease such as Alzheimer's disease, Parkinson's disease or Amyotrophic Lateral Sclerosis, dying neurons display morphological features of apoptosis (reviewed in Jellinger, 2003).

In particular, a role for apoptosis in Alzheimer's disease is supported by evidence from studies performed *in vivo* and *in vitro*. Several studies showed that dying neurons in Alzheimer's disease brains display apoptotic features (Su et al., 1994; Cotman et al., 1995; Colurso et al., 2003; Guo et al., 1999; Rohn et al., 2001). DNA fragmentation is significantly increased in neurons of Alzheimer's disease brains compared to normal brains (Colurso et al., 2003), and Bcl-2 is upregulated in brains of Alzheimer's disease patients (Su et al., 1996). In addition, immunohistochemical studies demonstrated caspase activation in damaged cells in Alzheimer's disease brains (Masliah et al., 1998; Su et al., 2000; Rohn et al., 2001, 2002).

In vitro studies indicate that Aβ causes neuronal death by a mechanism characterized as apoptosis (Nakagawa et al., 2000; Pike et al., 1993). Aβ induces apoptosis in cultured cortical (Loo et al., 1993; Allen et al., 1999), hippocampal (Jordan et al., 1997) and basal forebrain cholinergic neurons (Jhamandas and MacTavish, 2004). In cultured neurons expressing mutant forms of the APP and the presenilin 1 genes, an increase of Aβ level was observed and apoptosis was evident (Kwok et al., 2000; Guo et al., 1999).

The evidence that caspase activation was induced by exposure of neurons to Aβ together with the fact that caspase inhibition attenuates cell death, support the idea that caspase activation play an essential role in neuronal death in the context of Alzheimer's disease (Ivins et al., 1999; Giovanni et al., 2000; Troy et al., 2000; Harada et al., 1999; Nakagawa et al., 2000).

With respect to the identity of caspases involved in Alzheimer's disease-related neuronal death, caspase 3 functions as the principal effector of neuronal apoptosis in Alzheimer's disease brains (reviewed in Shimohama, 2000; Yuan and Yankner, 2000; Rohn et al., 2001; Takuma et al., 2004). Activation of caspase 2, 6, 8 and 9 (Engidawork et al., 2001; Guo et al., 2004; Rohn et al., 2001, 2002) has also been detected. In addition, exposure of cultured neurons to Aβ results in the activation of caspase 2, 3, 8 and 12 and inhibition of caspases attenuates Aβ-induced cell death (Ivins et al., 1999; Giovanni et al., 2000; Troy et al., 2000, Nakagawa et al., 2000).

Besides caspase activation, Aβ is also able to activate other apoptosis-related mechanisms involving c-Jun Kinase (Troy et al., 2001) and oxidative stress (Butterfield et al., 1999). Moreover, Selznick et al. (2000) reported that Aβ induces neuronal death by a mechanism dependent on Bax but independent of caspases.

In opposition to the evidence for the involvement of apoptosis in neuronal death, Geddes and collaborators failed to find signs of apoptosis in Alzheimer's disease brains, and interpreted that neurons die by non-apoptotic mechanism (Geddes et al., 1996). However it is important to note that some morphological hallmarks of apoptosis could have been lost in post-mortem samples due to rapid phagocytosis by neighboring macrophages or glial cells.

1.4.3. Axonal Degeneration in Alzheimer's disease

In addition to the apoptotic process that operates in the nucleus, neurons have self-destructive programs that localize to axons. These mechanisms are activated in response to local insults and take place in different pathological conditions (Raff et al., 2002; Coleman and Perry, 2002). Axonal degeneration is common in chronic neurodegenerative disorders (Lunn et al., 1989; Klucken et al., 2003; Bartzokis et al., 2004; Azzouz et al., 1997) and might occur early in the disease process, contributing to the symptoms and signs (Coleman and Perry, 2002; Adle-Biassette et al., 1999). In most cases however, it is unclear whether axonal degeneration is a consequence of neuronal apoptosis, or conversely whether axonal degeneration is the primary event leading to neuronal death. Moreover, the exact molecular mechanisms underlying axon degeneration are poorly understood.

Axon and synapse pathology has been demonstrated in Alzheimer's disease brains (Onorato et al., 1989; Coleman and Yao, 2003), and axonal degeneration and swelling is present in animal models of Alzheimer's disease (Probst et al., 1991; Bian et al., 2002). Nevertheless, the significance of axonal pathology in Alzheimer's disease is often underestimated (Coleman and Ribchester, 2004). The relevance of synaptic dysfunction, on the other hand, is well accepted (Oddo et al., 2003; Kita et al., 2000; Coleman and Ribchester, 2004; Scheff et al., 2003; Richardson et al., 2003).

1.4.4. Mechanisms of Axonal Degeneration

A classical example of axonal degeneration is Wallerian degeneration which occurs when an axon is cut (Waller, 1850). The distal segment of the axon responds to electrical stimulation for one or two days, and then rapidly degenerates. Wallerian degeneration has received considerable attention because morphological features associated with it, such as axonal beading and disintegration, are common to many neurodegenerative disorders or can be triggered by neurotoxins (Coleman and Perry, 2002; Cliffer et al., 1998). Wallerian degeneration was considered to be a passive mechanism of atrophy. However, this concept was changed by the discovery of the *Wld*^{S} (Wallerian gene) mutant mouse (Lunn et al., 1989), whose axons are protected from injury, toxicity and disease (Coleman and Perry, 2002). Evidence from *Wld*^{S} mutant mice indicate that axonal degeneration is an active process that occurs locally in the axons and is independent of cell body apoptosis (Conforti et al., 2000; Mack et al., 2001).

Recently, Zhai et al. showed that the ubiquitin-proteasome system is involved in Wallerian degeneration as well as in axon degeneration caused by NGF deprivation (Zhai et al., 2003). The ubiquitin-proteasome system is one of the main pathways of protein turnover. It is responsible for the clearance of damaged or misfolded proteins as well as for regulating normal protein turnover in order to control diverse cellular processes ranging from cell division to axon guidance (reviewed in Coleman, 2004). During Wallerian degeneration and NGF deprivation, inhibition of ubiquitin/proteasome system prevents axonal degeneration (Zhai et al., 2003; Macinnis and Campenot, 2005).

Caspase activation has also been implicated in axonal degeneration induced by different stimuli (Mattson et al., 1998a, b; Cowan et al., 2001; Ivins et al., 1998). In neurites of neurons from Alzheimer's disease brain, a breakdown product of actin produced by caspases has been detected by immunohistochemistry although caspase activation has not been directly demonstrated in neurites (Yang et al., 1998; Su et al., 2001). Ivins and colleagues (1998) have reported that neurite degeneration induced by local insults including Aβ, might involve local caspase activation in neurites since it is blocked by the caspase inhibitor zVAD-fmk (Ivins et al., 1998). In contrast, Finn et al. (2000) have shown that the general caspase inhibitor zVAD-fmk cannot prevent Wallerian degeneration of transected axons of the optic and sciatic nerve, nor can it prevent axonal degeneration induced by NGF deprivation in dorsal root ganglion neurons. Recent work from Campenot's laboratory supports these findings (Macinnis and Campenot, 2005).

An additional group of proteins implicated in neurodegeneration are calpains (Araujo Couto et al., 2004; Kieran et al., 2004; Nixon, 2003). Calpains belong to a family of Ca^{2+}-activated cystein proteases, important for diverse cellular functions including neuronal development, growth and apoptosis (reviewed in Suzuki et al., 2004). There are two major forms of calpain, μ- and m-calpain named according to the concentrations of Ca^{2+} necessary for their *in vitro* activation (micromolar and millimolar, respectively) (reviewed in Suzuki et al., 2004).

Increased calpain activity was found in Alzheimer's disease brains (Kuwako et al., 2002; Tseng et al., 2002; Adamec et al., 2002; Tsuji et al., 1998). Calpains are involved in apoptosis (Gao et al., 2000; Wood et al., 1998) and in the destruction of cytoskeletal proteins (Patrick et al., 1999; Glass et al., 2000). Glass et al. (2000) reported that calpain activation constitutes an early event in Wallerian degeneration of sciatic nerve and dorsal root ganglion neurons. Calpain activation was also reported in Wallerian degeneration after optic nerve crush and the calpain inhibitor, Mu-F-hF-FMK was able to preserve the cytoskeleton and axon integrity (Araujo Couto et al., 2004). Although there is increasing evidence that calpain activation is important in axonal degeneration and neuronal death, the exact role of calpains remains to be investigated.

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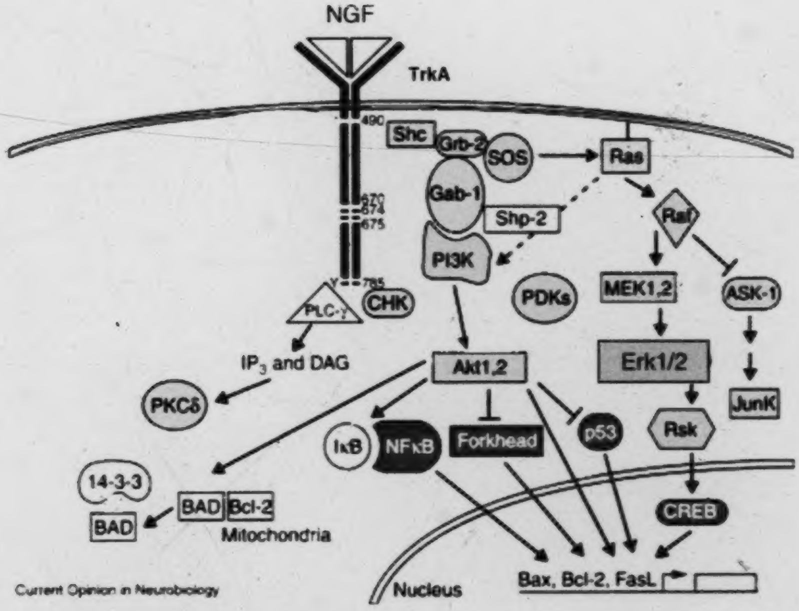
NGF
TrkA
490
Shc
Grb-2
SOS
Ras
Gab-1
Shp-2
670
674
675
PI3K
Raf
MEK1,2
ASK-1
Y
785
PLC-γ
CHK
PDKs
IP$_3$ and DAG
Akt1,2
Erk1/2
JunK
PKCδ
IκB
NFκB
Forkhead
p53
Rsk
14-3-3
BAD
Bcl-2
BAD
CREB
Mitochondria
Nucleus
Bax, Bcl-2, FasL
Current Opinion in Neurobiology

Figure1-1. Diagram of neurotrophin signal transduction pathways mediated by TrkA receptor. Adaptor proteins are colored orange, kinases are purple, small G proteins green and transcriptional factors blue. Three main pathways are indicated: Ras/Erk, PI3Kinase/Akt and PLCγ pathways. Adapted from Patapoutian and Reichardt (2001), *Curr Opin Neurobiol.*

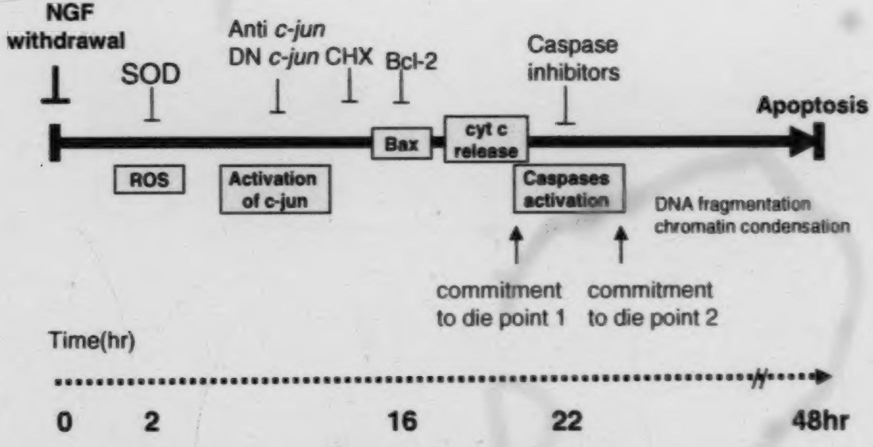
NGF
withdrawal
SOD
Anti c-jun
DN c-jun CHX
Bcl-2
Caspase
inhibitors
Apoptosis
ROS
Activation
of c-jun
Bax
cyt c
release
Caspases
activation
DNA fragmentation
chromatin condensation
commitment
to die point 1
commitment
to die point 2
Time(hr)
0
2
16
22
48hr

Figure1-2. Molecular events activated during apoptosis of cultured sympathetic neurons deprived of NGF. The temporal sequence of events activated by NGF withdrawal is represented in green boxes. Neuronal death depends on protein synthesis and is mediated by Bax. Other important events are boxed. Modified from Deshmukh et al.(1997) , Chang et al. (2002)

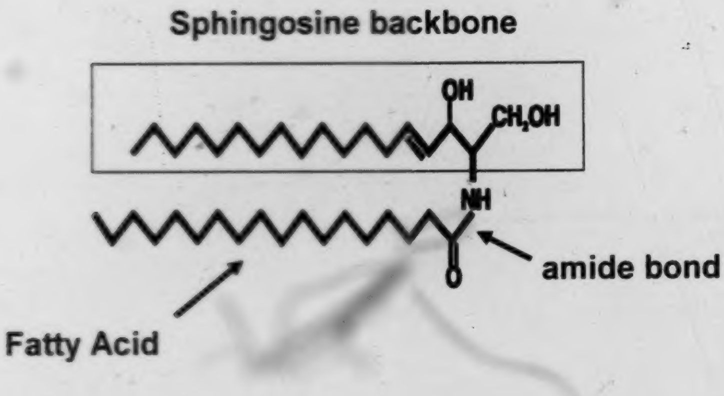
Sphingosine backbone
OH
CH2OH
NH
O
amide bond
Fatty Acid

Figure1-3. Structure of ceramide. Ceramide consists of an 18-carbon backbone of sphingosine linked by an amide bond to a variable fatty acid chain of 2-28 carbons.

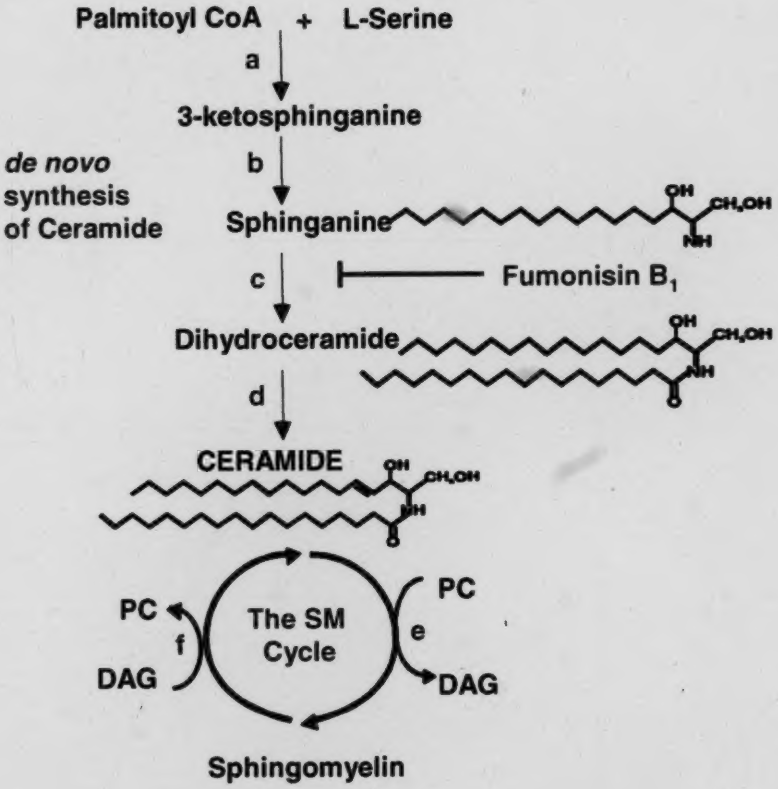
Palmitoyl CoA + L-Serine
a
3-ketosphinganine
de novo synthesis of Ceramide
b
Sphinganine
OH
CH2OH
NH
c
Fumonisin B1
Dihydroceramide
OH
CH2OH
NH
O
d
CERAMIDE
OH
CH2OH
NH
O
PC
f
DAG
The SM Cycle
PC
e
DAG
Sphingomyelin

Figure1-4. Ceramide Synthesis and Metabolism

a. Serine palmitoyl transferase
b. 3-ketosphinganine reductase
c. Sphinganine N-acyl transferase (ceramide synthase)
d. Dihydroceramide desaturase
e. Sphingomyelin synthase
f. Sphingomyelinase

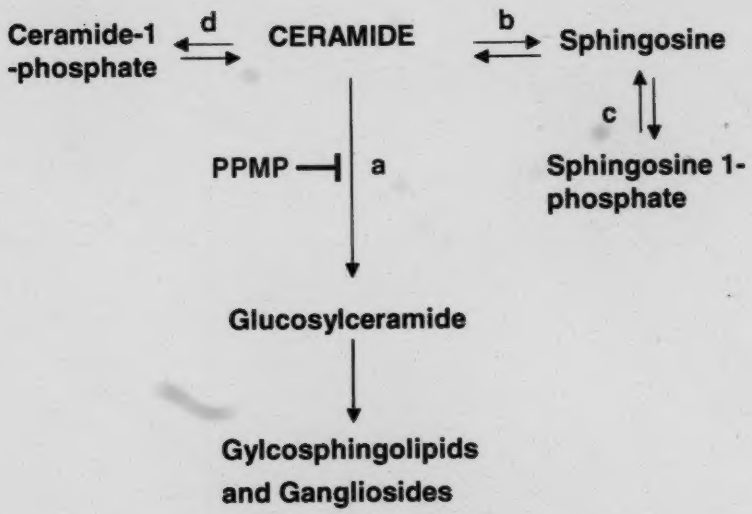
Ceramide-1
-phosphate
d
CERAMIDE
b
Sphingosine
c
Sphingosine 1-
phosphate
PPMP
a
Glucosylceramide
Gylcosphingolipids
and Gangliosides

Figure1-5. Ceramide Metabolism

a. Glucosylceramide synthase
b. Ceramidase
c. Sphingosine kinase
d. Ceramide kinase

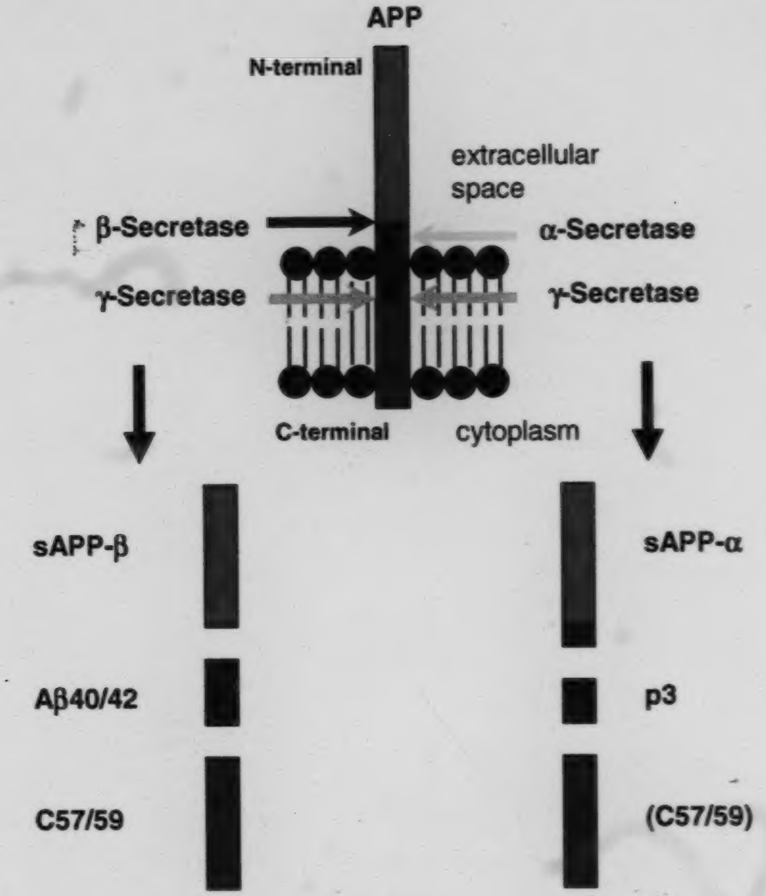
APP
N-terminal
extracellular space
β-Secretase
α-Secretase
γ-Secretase
γ-Secretase
C-terminal
cytoplasm
sAPP-β
sAPP-α
Aβ40/42
p3
C57/59
(C57/59)

Figure 1-6. APP processing. The major pathway of APP cleavage involves proteolysis by α-secretase at the N-terminus and γ-secretase at the C terminus. As a result sAPP-α and p3 are generated. APP also serves as a substrate for β-secretase which cleaves at the N-terminus generating sAPP-β and a C-terminal fragment which in turn is cleaved by γ-secretase to form Aβ.

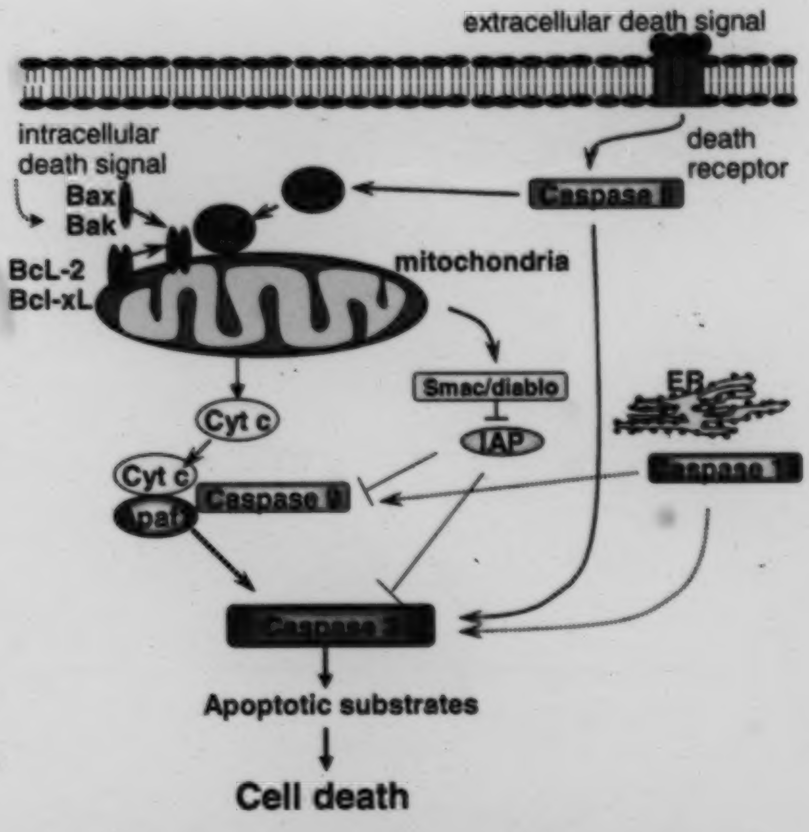
extracellular death signal
intracellular death signal
Bax
Bak
BcL-2
Bcl-xL
death receptor
Caspase 8
mitochondria
Smac/diablo
IAP
ER
Caspase 12
Cyt c
Cyt c
Apaf
Caspase 9
Caspase 3
Apoptotic substrates
Cell death

Figure 1-7. Molecular Pathways of Apoptosis. The death receptor (extrinsic) pathway is activated by ligand binding to death receptors(Fas/CD95), result in caspase-8 activation. Then caspase-8 can proteolytically activate caspase-3 to initiate degradation of various substrates. In addition, caspase-8 can cleave the pro-apoptotic BcL-2 family resulting in cytochrome c release from mitochondria. In the mitochondrial (intrinsic) pathway, other proteases (cytotoxic lymphocyte protease or lysosomal cathepsins) can proteolytically activate Bid, which then translocates to mitochondria, where it facilitates cytochrome c (cyt c) release. Other stimuli including DNA damage and toxins can induce cytochrome c. Modified from Vila and Przedborski (2003), *Nature Reviews Neuroscience*

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Trypsin, collagenase and *N*-oleoyl-ethanolamine (NOE) were obtained from Sigma-Aldrich Ltd. (Canada). Leibovitz L15-CO_2 culture medium was from Life Technologies Inc. (Canada). Mouse NGF (2.5S) and LY294002 were from Alomone Laboratories Ltd. (Israel). Human brain-derived neurotrophin factor (BDNF) was from Peprotech Inc. (Canada). Anti NGF antibody was purchased from Cedarlane Laboratories Ltd. (Canada). K252a and Calpastatin peptide were purchased from Calbiochem (San Diego, CA, USA). C_6-ceramide, C_6-dihydroceramide (C_6-DHcer), standard rat brain ceramides, *threo*-DL-1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) and *erythro*-PPMP were obtained from Matreya Inc. (Pleasant Gap, PA). Fumonisin B_1 (FB_1) was provided by Alexis Corp. (San Diego, CA, USA). Diacylglycerol kinase (DAG-kinase) (*E. coli*), was a kind gift from Dr. D. Brindley (University of Alberta). [9,10-^{3}H]Palmitate (specific activity 54 Ci/mmol), [*methyl*-^{3}H]choline chloride (80 Ci/mmol) and [γ^{32}P]ATP (3000 Ci/mmol) were supplied by Amersham (Canada). Thin layer chromatography plates (silica gel G) were obtained from BDH Chemicals (Canada). Standard phospholipids were isolated from rat liver. Rat serum and rat-tail collagen were prepared as before (de Chaves et al., 1997). Teflon dividers were provided by Tyler Research Instruments (Edmonton, Canada). Immobilin polyvinylidene difluoride (PVDF) membranes used for immunoblotting were purchased from Bio-RAD (Hercules, CA, USA). Enhanced chemiluminescence reagents used for detection of immunoreactivity were prepared in our laboratory. Secondary antibodies used for immunoblotting were from Pierce (Brockville, Ont., Canada). Alexa Fluor 488- and Alexa Fluor 594-conjugated goat anti-rabbit and anti-mouse secondary antibodies used for immunocytochemistry were from Molecular Probes (Eugene, OR, USA).

$A\beta_{1-42}$ and its reverse peptide $A\beta_{42-1}$ were purchased from American Peptide Company (Sunnyvale, CA, USA). BOC-D-fmk (BOC-Asp (OMe)-Fluoromethyl ketone) was obtained from Kamiya (Seattle, WA, USA) and MG132 was from Biomol (Plymouth Meeting, PA, USA). Cell Titer 96 Cell Proliferation Assay (MTT) was from Promega

(Madison, WI, USA). All other reagents were from Sigma-Aldrich (Oakville, Ont., Canada) or Fisher Scientific (Edmonton, Alta., Canada).

2.2. Methods

2.2.1. Culture of neurons

Sympathetic neurons isolated from superior cervical ganglia of newborn Harlan Sprague–Dawley rats (Health Science Lab Animal Services, University of Alberta) were isolated by enzymatic dissociation with 1% (w/v) of collagenase and 0.1% (w/v) of trypsin followed by mechanical dissociation using a flame-polished Pasteur pipet. After filtration of the cell suspension through cell strainer (40 µm Nylon; Falcon, Becton Dickinson, NJ, USA), the cell suspension was plated into culture dishes. The standard culture medium was L15 CO_2 supplemented with the prescribed additives (Hawrot, 1979) and 0.4% methylcellulose. Non-neuronal cells were eliminated by supplying 10–15 µM cytosine arabinoside during the first 5–6 days in culture.

Two types of neuronal cultures were used. Mass cultures in which neurons were plated in 96-well dishes at a density of 0.16 ganglia/well or 24-well dishes at a density of 1–2 ganglia/well were used for apoptosis detection and immunoblot analysis, respectively. Medium supplied to mass cultures was supplemented with 2.5% rat serum, 1 mg/ml ascorbic acid and 50 ng/ml NGF. Alternatively, sympathetic neurons were cultured in three-compartment culture dishes (de Chaves et al., 1997) (Fig 2-1). Compartmented cultures were constructed as previously described (Campenot, 1982). Briefly, collagen was dried onto 35-mm culture dishes. Parallel scratches were made in the collagen substratum and a Teflon divider was attached to the collagen surface with silicone grease. The dissociated neurons (0.2 ganglia per dish) were plated in the center compartment. Within 1–2 days axons grew along the tracks and entered the side compartments that contained medium supplemented with 50 ng/ml NGF. Medium supplied to the center compartment contained 2.5% rat serum, 1 mg/ml ascorbic acid, 10–15µM cytosine arabinoside and 10 ng/ml NGF. After 5–6 days cytosine arabinoside treatment was discontinued and NGF was confined to the side compartments.

Rat basal forebrain cholinergic neurons (BFCN) were generously provided by Dr. Jhamandas (University of Alberta) and were prepared as described previously (Jhamandas and MacTavish, 2004). Briefly, septal regions containing the basal forebrain neurons were dissected in Hanks balanced salt solution supplemented with 15 mM HEPES, penicillin, and streptomycin; trypsinized followed by trituration and then plated on a 96-well plate (3×10^3 per well) coated with 10 mg/ml poly-D-lysine. The cultures were grown at 37°C in a 5% humidified atmosphere in Neurobasal medium supplemented with N-2. All experiments started at day 7–8 in culture.

2.2.2. NGF deprivation experiments

Sympathetic neurons in mass cultures were maintained in medium containing 50 ng/ml NGF for 7–8 days. At that time neurons were rinsed 3 times with basal medium lacking NGF followed by 10 min incubation with the same medium. To ensure that no NGF was available during deprivation, medium containing 24 nM sheep anti-NGF antibody (α-NGF) was used. To create a set of cultures deprived of NGF for various periods between 0 and 48 h, the onset of NGF deprivation was varied accordingly. Survival agents (C_6-ceramide, sphingosine, cycloheximide, etc.) were added to the medium containing α-NGF. Control cultures were given 50 ng/ml NGF throughout the experiment. To control for the effect of changing the medium, at any particular time all cultures were processed in parallel. For deprivation experiments in compartmented cultures a similar protocol of rinsing and α-NGF treatment was followed. Control cultures were maintained with NGF exclusively in distal axons by providing 24 nM α-NGF in the cell body-containing compartment and 50 ng/ml NGF in the distal axons-containing compartments. Neuronal survival under these conditions was maximal and the same as in cultures given 10 ng/ml NGF in the center compartment and 50 ng/ml NGF in the side compartments. In experiments in which PPMP was used, neurons were treated with PPMP (*threo* or *erythro* isomer as indicated) for 3 days previous to NGF deprivation as before (de Chaves et al., 1997). PPMP was also supplied to the medium containing α-NGF after deprivation.

2.2.3. Commitment-to-die point experiments

Mass cultures were deprived of NGF by adding α-NGF. At various times thereafter, 50 ng/ml NGF, 30 μM C_6-ceramide or 1 μg/ml cycloheximide was added to appropriate wells. Media with C_6-ceramide or cycloheximide also contained α-NGF. After an additional 2 day-period, cultures were fixed and analyzed for apoptosis. The results are expressed as percentage of apoptotic neurons. For a better comparison between different agents and since the protection from apoptosis by most agents (except NGF) is not complete, I calculated the percentage of survival at each time in reference to the survival with that particular agent when added at time 0 h. The commitment point is represented by the time at which survival is 50%.

2.2.4. Oligomerization and Fibrillization of Aβ $_{1\text{-}42}$

Oligomeric Aβ (oAβ) and fibrillar Aβ (fAβ) were prepared according to published protocols (Dahlgren et al., 2002). Briefly, $A\beta_{1\text{-}42}$ peptide was dissolved initially to 1 mM in hexafluoroisopropanol and separated into aliquots in sterile microcentrifuge tubes. Hexafluoroisopropanol was dried under a stream of N_2 and the peptide films were dessicated at -20°C. The peptide was resuspended in DMSO (Me_2SO) to a concentration of 5 mM. To obtain oAβ, Leibovitz L15-CO_2 medium (phenol red-free, antibiotics free, serum free) was added to bring the peptide to a final concentration of 100 μM and incubated at 4 °C for 24h. To obtain fAβ, 10 mM HCl was added to bring the peptide to a final concentration of 100 μM and incubated for 24 h at 37 °C. To use Aβ in the soluble, monomeric form (mAβ), 5 mM Aβ in DMSO was diluted directly into cell culture media.

2.2.5. Treatment of neurons with Aβ

Sympathetic neurons were cultured in 3-compartment dishes for 7-8 days. At that time Aβ prepared as indicated above was added to the medium provided to the side, distal axons-containing compartments (designated DAX hereafter). Control cultures received vehicle alone. Unless indicated cultures were provided with medium without NGF in the center, cell body-containing compartment (designated CB hereafter). For cultures deprived of NGF, medium without NGF was given to all three compartments. To ensure that no NGF was available during deprivation, medium containing sheep anti-NGF antibody (α-NGF: 8 nM) was used. Basal forebrain cholinergic neurons and sympathetic neurons cultured in 96 well dishes were provided with Aβ in regular culture medium.

2.2.6. Detection of apoptotic cell death

Apoptotic cell death was examined by nuclear staining with Hoechst 33258 and by *in situ* activation of caspase-3. For Hoechst staining, sympathetic neurons were fixed with 4% paraformaldehyde in PBS for 20 min and stained with 500 ng/ml Hoechst 33258 for 10–20 min. The percentage of apoptotic neurons was determined by counting condensed and/or fragmented nuclei versus evenly stained nuclei. Unless specified, the total numbers of apoptotic neurons include ghost cells. These ghost cells represent neurons in the late stage of apoptosis. They are visible under phase contrast microscopy but devoid of chromatin (Edwards et al., 1994). Activation of caspase-3 *in situ* was evaluated by immunocytochemistry using anti-cleaved caspase-3 (Asp 175) antibody (1:100) (Cell Signaling Technology, Beverly, MA) as primary antibody and FITC-labeled secondary antibody according to a protocol similar to the one described below under immunocytochemistry. Simultaneously nuclei were stained with Hoechst 33258. Immunoreactivity to caspase-3 was detected by microscopic analysis and the percentage was calculated by counting positive and negative neurons. Neurons were visualized using a Nikon TE300 inverted fluorescence microscope equipped with a Nikon digital camera DXM-1200 (Nikon Canada, Toronto, Ont.). Images were analyzed using Northern Elite V6.0 image capture and analysis software (Empix Imaging, Missisauga, Ont.). Five hundred to 1000 neurons per treatment were counted in 3-5 wells by an observer "blinded" to the neuronal treatment. Results were expressed as means ± SEM of at least 3 experiments. Data were analyzed using the Kruskal–Wallis test with post hoc comparison test. The level of statistical significance selected was a *P*-value <0.05.

Simultaneously, for experiments in which nuclear apoptosis and axonal viability were examined in compartmented cultures (Chapter 4), cell bodies in the center compartment were fixed immediately after the cellular materials from the distal axons-containing compartment were collected.

2.2.7. Evaluation of axonal viability

Axonal degeneration was evaluated using the MTT reduction assay (Promega). This assay is based on the conversion of MTT (3-(4,5-dimethylthiozolyl)-2,5-diphenyltetrazolium bromide) from a yellow to a blue formazan crystal by NADPH or NADH produced by dehydrogenase enzymes in metabolically active cells (Berridge et al., 1993). Neurons were treated according to each experiment. At the end of the treatment, MTT was added to the medium given to distal axon-containing compartments and incubated for 1 hr in the CO_2 incubator at 37° C. The blue formazan product of MTT reduction was a) visualized under the microscope and b) quantitatively measured. Direct observation of decreased color formation in bright field images is indicative of decreased axonal metabolic activity and therefore viability. For quantification purposes the reaction was stopped with stop/solubilization solution and the solubilized, colored compound from distal axons was transferred to a 96-well plate. The absorbance at 570nm was recorded (Multiskan Plus MK II). The absorbance obtained with untreated axons was considered as 100% viability. All other values were referred to this control.

2.2.8. Incorporation of [^{3}H]palmitate into ceramide

Neurons grown for 7 days in 24-well dishes were treated in the presence of 10μCi/ml [^{3}H]palmitate with or without 30μM C_6-ceramide for 24 h. In some cultures Fumonisin B_1 (FB_1) (25 or 50μM) was added. Some other cultures received 30μM C_6-DHcer. Radiolabeled medium was aspirated, and neurons were washed twice with ice-cold phosphate-buffered saline (pH 7.4). Cellular material was collected in methanol/water (1:1, v/v) and sonicated for 10 s using a probe sonicator. Chloroform was added to give a final ratio of chloroform/methanol/water of 2:1:1 (v/v). Ceramide was separated by thin layer chromatography as before (de Chaves et al., 1997). The bands corresponding to ceramide were scraped, and radioactivity incorporated was measured. Radioactive incorporation was normalized to total phospholipid mass.

2.2.9. Determination of Sphingomyeline levels

Neurons cultured in 24-well dishes were incubated with medium containing 1μCi/ml [^{3}H]*methyl*-choline chloride for 3 days. At the end of the labeling period, the cells were washed extensively with base medium. Neurons were stimulated with 50 ng/ml NGF or 100 ng/ml BDNF. Some cultures that received NGF were preincubated with 200 nM K252a for 1 h and received K252a thereafter. For cultures incubated with BDNF a medium containing α-NGF was used. After the indicated times cellular material was harvested and lipids were isolated (Folch and Sloane-Stanley, 1959). Sphingomyeline content was determined with bacterial SMase. The results are expressed as percentage of control of neurons given 50 ng/ml NGF.

2.2.10. Measurement of mass of ceramide

Cultures were treated as for determination of SM levels except for the labeling with [^{3}H]*methyl*-choline chloride. Lipids were extracted by the Bligh–Dyer method and mass amounts of ceramide in cellular extracts were measured by a modification of the DAG-kinase enzymatic method (Abousalham et al., 1997). The mass of ceramide was calculated based on radioactivity of a standard curve processed in parallel. The results are expressed in reference to the mass of total phospholipids.

2.2.11. Immunocytochemistry

Neurons cultured in 96-well dishes were washed twice with ice-cold Tris-buffered saline (TBS), fixed with 4% paraformaldehyde on ice for 20 min and incubated with blocking buffer (2% bovine serum albumin, 0.2% non-fat milk, 2% normal goat serum, 0.4% Triton X-100 in TBS) at room temperature for 1 h. Primary antibodies anti-pAkt

(Ser473) (1:50) (Promega Corp., Madison, WI, USA) or anti-cleaved caspase-3 (Asp 175) antibody (1:100) (Cell Signaling Technology, Beverly, MA) prepared in blocking buffer were applied for 1 h at room temperature. After washing with 0.4% Triton X-100 in TBS, neurons were incubated with blocking buffer containing AlexaFluor 488 (green fluorescence) IgG or AlexaFluor 594 (red fluorescence) secondary antibody (1:50) (Molecular Probes, Eugene, OR, USA) and 500 ng/ml Hoechst 33258 for 1 h at room temperature. The preparations were mounted with 3.3% *N*-propyl-gallate and 0.6% glycerol in TBS to prevent fluorescence quenching. Immunoreactivity was analyzed using a Nikon TE300 inverted fluorescence microscope as indicated above. Five hundred to 1000 neurons per treatment were counted by an observer "blinded" to the neuronal treatment.

2.2.12. Immunoblotting

Sympathetic neurons cultured in 24-well plates were washed with ice-cold TBS containing 1 mM sodium orthovanadate and 10 mM sodium fluoride. Cellular material from 2 wells of the same treatment was harvested with modified Laemlli sample buffer (40 mM Tris–HCl pH 6.8, 1% sodium dodecyl sulfate (SDS), 4% 2-mercaptoethanol, 10% glycerol and 0.002% bromophenol blue) and boiled for 2 min. Proteins were separated by SDS-PAGE on 8% polyacrylamide gels containing 0.1% SDS and transferred overnight at 4 °C to PVDF membranes in 25 mM Tris: 192 mM Glycine, 16% methanol buffer, pH 8.3, containing 0.1% SDS. Membranes were blocked for 1 h in TBS, 0.1% Tween-20 (TTBS) containing 5% non-fat milk and incubated overnight in the primary antibody solution prepared in TTBS containing 5% bovine serum albumin. In the case of anti-p75^{NTR}, this incubation was performed for 1 h at room temperature. The following primary antibodies were used: rabbit polyclonal anti-phospho TrkA (Tyr490) (1:1000), anti-phospho Akt (S473) (1:1000) and anti-phospho p44/42 MAPK (Thr202/Tyr204) (ERK 1/2) from Cell Signaling Technology (Beverly, MA, USA); affinity purified anti-pan-Trk polyclonal antibody Trk (C-14), (1:250) from Santa Cruz Biotechnology (Santa Cruz, CA), rabbit polyclonal anti-human p75^{NTR} (1:1000) (Promega Corp., Madison, WI, USA) and monoclonal anti-α-, β-tubulin antibodies

(1:2000) from Sigma-Aldrich (Oakville, Ont., Canada). Primary antibodies for cleaved caspase-9 (1:1000), cleaved caspase-3 (1:1000) and ubiquitin (1:2000) were from Cell Signaling Technology (Beverly, MA, USA). Membranes were washed three times with TTBS and then incubated for 1 h with the secondary antibody (1:2000) in blocking buffer at room temperature with gentle agitation. For the detection of α-, β-tubulin and ubiquitin, secondary antibody (1:5000) was prepared in 0.1% TTBS instead of blocking buffer. Immunoreactivity was detected by Enhance Chemo Luminescence (ECL). Equal loading was checked by probing for α-, β- tubulin.

2.2.13. Other methods

The phospholipid content of cells was measured by lipid phosphorus determination (Chalvardjian and Rudnicki, 1970).

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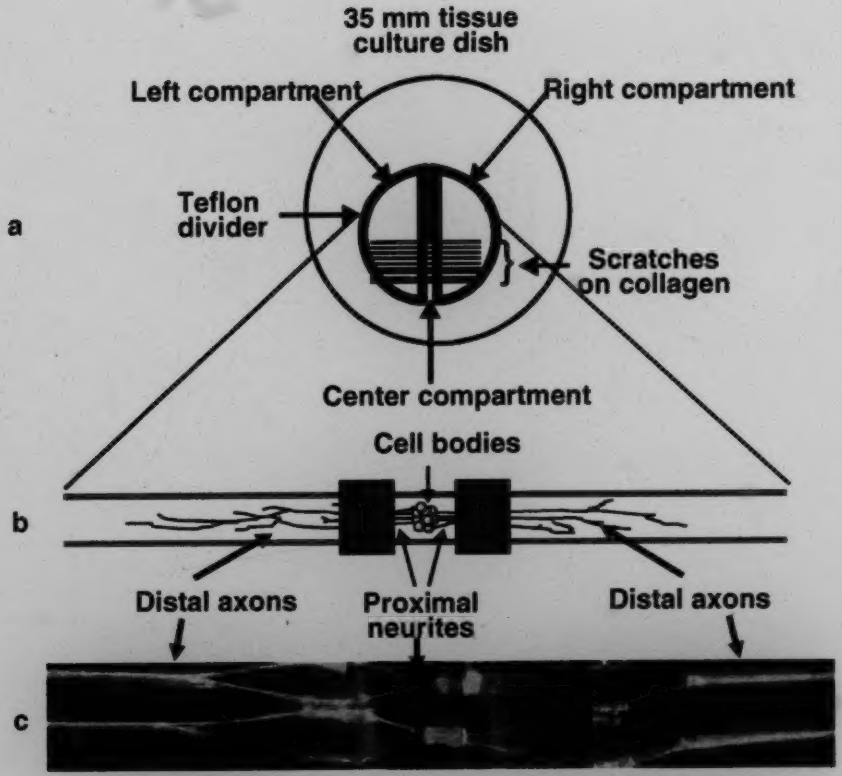
35 mm tissue culture dish
Left compartment
Right compartment
a
Teflon divider
Scratches on collagen
Center compartment
Cell bodies
b
Distal axons
Proximal neurites
Distal axons
c

Figure 2-1. The three-compartment model for culture of sympathetic neurons. Shown is a representation of a compartmented culture. *a* illustrates an entire culture, and *b* is an enlargement of a single track of a culture in which neurons are plated in the center compartment. Neurites extend to the left and right, under silicone grease barriers, and into the separate fluid environments of left and right compartments. *c* is a composed micrograph of a single track of a culture in which neurons have been labeled with a lipophilic fluorescent dye.

CHAPTER 3

Characterization of the Antiapoptotic Effect of Ceramides in Rat Sympathetic Neurons –Identification of the Molecular Mechanisms Underlying the Inhibition of Apoptosis

Most of the data presented in this chapter were published in: Song MS and Posse de Chaves EI. 2003. Inhibition of rat sympathetic neuron apoptosis by ceramide. Role of p75^{NTR} in ceramide generation. *Neuropharmacology.* 45(8):1130-50.

3.1. Introduction

In the past decade major findings have emphasized the importance of ceramide as a bioactive molecule. Ceramide is an essential mediator of cell growth and stress response (Hannun and Luberto, 2000; Pfeilschifter and Huwiler, 2000); modulation of ceramide biosynthetic pathways has therapeutic potential (Claus et al., 2000; Kolesnick, 2002; Radin et al., 2001) and ceramide may be crucial in the effectiveness of various cancer treatments (Perry, 2000; Senchenkov, 2001). Several agents and stress stimuli cause elevation of ceramide (Hannun, 1996) and induce different and sometimes opposing effects depending on the cell type. The most comprehensively studied is the role of ceramide in apoptosis (Hannun and Luberto, 2000; Kolesnick and Kronke, 1998).

The discovery that neurotrophins, binding to the p75 neurotrophin receptor ($p75^{NTR}$) stimulate sphingomyelin hydrolysis with concomitant elevation of ceramide (Dobrowsky et al., 1994) underscored the relevance of ceramide as a regulator of neuronal death. The evidence for $p75^{NTR}$-dependent ceramide production extended to several systems (Brann et al., 1999, 2002 Casaccia-Bonnefil et al., 1996; DeFreitas et al., 2001 ; Dobrowsky et al., 1994) and as a consequence ceramide is often considered a downstream effector of $p75^{NTR}$ (Barrett et al., 2000; Mamidipudi et al., 2002). Data supporting such assumption in primary sympathetic neurons, however, are still missing. In sympathetic neurons, ceramide inhibits axonal elongation (de Chaves et al., 1997; de Chaves et al., 2001) and appears to protect from apoptosis caused by nerve growth factor (NGF) deprivation (Ito et al., 1995; Nair et al., 2000; de Chaves et al., 2001).

The present studies aim to: investigate the mechanism(s) involved in the inhibition of apoptosis by ceramide and examine the role of $p75^{NTR}$ in the generation of ceramide in sympathetic neurons. I demonstrate that C_6-ceramide is converted to long-chain ceramides and that endogenous ceramides as well as short-chain ceramide support the survival of sympathetic neurons in the absence of NGF. The results presented here indicate that the mechanism of survival support by ceramide involves phosphorylation of TrkA and selective activation of the phosphatidyl inositol 3-kinase (PI3-kinase)/Akt pathway. Using a compartmented culture system, I demonstrate that delivery of C_6-

ceramide exclusively to distal axons is sufficient to induce neuronal survival. This finding supports the concept that retrograde survival by TrkA activation can occur without the necessity of NGF retrograde transport. The simplicity of our model offers an advantageous option to study alternative mechanisms involved in target-driven neuronal survival. In addition, I demonstrate that stimulation of p75^{NTR} causes transient accumulation of ceramide, but the pool of ceramide generated is not involved in neuronal survival/death.

3.2. Results

3.2.1. C$_6$-ceramide as well as endogenous ceramide suppresses the death program initiated by NGF deprivation

It has been reported previously that ceramide inhibits cell death in cultured NGF-deprived sympathetic neurons (de Chaves et al., 2001; Ito et al., 1995; Nair et al., 2000), however previous reports failed to investigate whether ceramide was simply blocking cell death or was able to induce survival. In addition, in the only study in which apoptosis was evaluated and the mechanism of ceramide protection was analyzed, only limited protection against apoptosis was found (Nair et al., 2000). Therefore, in order to determine if ceramide induces survival of sympathetic neurons, I performed classical experiments of NGF deprivation over a period of 46–48 h in the presence or absence of ceramide. Seven to eight day-old sympathetic neurons were deprived of NGF and given neutralizing anti-NGF antibodies (α-NGF). I provided a number of the NGF-deprived cultures with 30 μM C$_6$-ceramide or the relatively inactive isomer C$_6$-DHCer. I selected the concentration of C$_6$-ceramide based on a dose–response curve (Plummer et al., 2005). Apoptosis was evaluated by nuclear staining with Hoechst 33258, a chromatin stain that reveals nuclear condensation and fragmentation associated with apoptotic nuclei. In parallel, I examined activation of caspase-3 "*in situ*" by immunocytochemistry using an antibody that is specific for the cleaved, catalytically active form of caspase-3 (Kuruvilla et al., 2000). As shown previously (Deckwerth et al., 1993), after 22–24 h of NGF deprivation ~50% of the neurons showed signs of apoptosis and more than 80% of the neurons died after 36 h in the absence of NGF (Fig. 3-1A). Addition of the short-chain ceramide analogue C_6-ceramide at the time of NGF deprivation greatly reduced the number of neurons undergoing apoptosis. Only ~30% of the nuclei from ceramide-saved neurons appeared condensed and/or fragmented and most neurons were morphologically indistinguishable from NGF-maintained neurons even after 46 h of NGF withdrawal (Fig. 3-1A and B). The protective effect of C_6-ceramide is specific since the inactive analogue C_6-DHCer did not offer any protection from apoptosis. C_6-ceramide needed to be present during the NGF deprivation period since treatment with ceramide prior to (up to 24 h) but not during NGF withdrawal was not effective (data not shown). Neither C_6-ceramide, nor C_6-DHCer, had any effect on survival when added in the presence of NGF for up to 7 d (de Chaves et al., 1997). Similar results were obtained using C_2-ceramide (not shown). Since sympathetic neurons contain endogenous C_2-ceramide but not C_6-ceramide (see below), I decided to use C_6-ceramide for further studies to be able to distinguish ceramides from exogenous and endogenous origins.

The main reason for using short-chain ceramides to study the role of ceramides as lipid second messengers is their greater ability to disperse in water and to be cell permeable. The use of short-chain ceramides has been justified in part by the finding that C_2-ceramide is a natural constituent of mammalian cells and by the fact that many effects attributed to cellular ceramide could be replicated by treatment with short-chain analogues. In some cases however, the effects of short-chain ceramides do not correspond to natural ceramides (reviewed in Ghidoni, 1999). Therefore, I decided to increase endogenous ceramide levels by using the inhibitor of ceramide metabolism, PPMP. The effectiveness of *threo*-PPMP but not *erythro*-PPMP in inhibiting the conversion of ceramide into complex glycosphingolipids in sympathetic neurons has been previously demonstrated (de Chaves et al., 1997). Treatment of sympathetic neurons with *threo*-PPMP but not *erythro*-PPMP causes accumulation of endogenous ceramides and produces biological effects equivalent to short-chain ceramides on axonal growth (de Chaves et al., 1997). To evaluate whether endogenous ceramides were also able to protect sympathetic neurons from apoptosis, neurons were treated with *threo*-PPMP or *erythro*-

PPMP for 3 days prior to NGF deprivation. This preincubation is necessary to obtain high levels of endogenous ceramides (de Chaves et al., 1997). The inhibitors were also included during the NGF deprivation period. In neurons deprived of NGF for 36 h, *threo*-PPMP was as effective as C_6-ceramide in suppressing apoptosis (Fig. 3-1B c–h and Fig. 3-1C). As expected, treatment with *erythro*-PPMP did not protect sympathetic neurons (Fig.3-1C). In the presence of NGF, neuronal survival was not affected by either of the isomeric forms of the inhibitor (see below: Fig 3-8D). Together these experiments show for the first time that endogenous ceramides are as effective as short-chain ceramide in inhibiting the apoptotic program triggered by NGF deprivation and to promote survival of sympathetic neurons in the absence of their natural neurotrophin.

3.2.2. C_6-ceramide is converted to long-chain ceramides in sympathetic neurons

Since C_6-ceramide can be converted into long-chain ceramides within cells (Gomez-Munoz, 1995; Ogretmen et al., 2002) some effects of the short-chain analogue could be attributed to elevation of long-chain ceramides. To examine the generation of endogenous long-chain ceramides from exogenously added C_6-ceramide, I treated sympathetic neurons with or without C_6-ceramide or C_6-DHCer in the presence of ^{3}[H]palmitate, a precursor for newly synthesized sphingolipids. I observed a significant increase in the incorporation of ^{3}[H]palmitate into ceramide in C_6-ceramide-treated neurons but not in C_6-DHCer-treated neurons after 24 h (Fig. 3-2A). This increase was completely abolished by Fumonisin B_1 (FB_1), a mycotoxin that inhibits the enzyme ceramide synthase blocking the acylation step in ceramide synthesis. Our results indicate that treatment of sympathetic neurons with 50 μM Fumonisin B1 not only eliminated ceramide effect but caused a further decrease in ^{3}[H]palmitate incorporation in comparison to NGF-treated neurons, likely by inhibition of the *de novo* ceramide synthesis. I corroborated the stimulation of long-chain ceramides synthesis by C_6-ceramide by measuring directly the mass of long-chain ceramides in neurons treated with or without C_6-ceramide or C_6-DHCer as before. I found that basal levels of long-chain ceramides in sympathetic neurons cultured with NGF are higher than basal levels found in other cell types (~100 pmol/nmol PL in sympathetic neurons vs. ~10–20 pmol/nmol

PL in fibroblasts). Nevertheless, treatment with C_6-ceramide caused a 2-fold increase in endogenous ceramides containing acyl chains equal or longer than 16 carbon atoms (Fig. 3-2B). C_6-DHCer did not significantly increase endogenous long-chain ceramides. The lack of conversion of C_6-DHCer into long-chain ceramides has been interpreted as lack of deacylation of this isomer (Ogretmen et al., 2002). As before, C_6-ceramide given to neurons together with Fumonisin B1 did not cause any increase in long-chain ceramide mass. Furthermore, as suggested by the experiments with ^{3}[H]palmitate, Fumonisin B_1 inhibited de novo ceramide synthesis and caused a decrease in the total mass of long-chain ceramides. With respect to cellular ceramides with shorter chains, I could detect C_6-ceramide only in neurons treated with the short-chain ceramide analogue suggesting an exogenous origin. On the other hand I detected endogenous C_2-ceramide in both treated and untreated neurons (see below). This finding that C_6-ceramide is converted into long-chain ceramides raises the question whether the effects of C_6-ceramide on survival were due to the short-chain analogue or to the long-chain endogenous ceramides.

3.2.3. C_6-ceramide inhibits apoptosis by means of the short analogue and the endogenous long-chain ceramides

In order to differentiate and separate the effects of C_6-ceramide due to the short analogue itself and to the long-chain endogenous ceramides, I asked if a 2-fold increase in the levels of endogenous ceramides generated by C_6-ceramide would be sufficient to inhibit apoptosis. To address this problem, I examined the levels of endogenous long-chain ceramides achieved by treatment with *threo*-PPMP under conditions in which apoptosis is inhibited. It was previously demonstrated that *threo*-PPMP but not *erythro*-PPMP induces an increase in ^{3}[H]palmitate incorporation into ceramide (de Chaves et al., 1997). Here, I measured the mass of ceramide by the diacyglycerol-kinase assay. I treated the neurons for 3 d with *threo*-PPMP or *erythro*-PPMP; some cultures were pretreated for 24 h with Fumonisin B_1 previous to PPMP. *threo*-PPMP but not *erythro*-PPMP caused ~2.5-fold increase in long-chain endogenous ceramides (Fig. 3-3A). The mass of endogenous long-chain ceramides in cultures treated with *threo*-PPMP was consistently higher than in C_6-ceramide-treated neurons however the difference was not statistically significant ($p>0.05$). Since ceramides accumulated by *threo*-PPMP come exclusively from the *de novo* pathway, Fumonisin B_1 abolished ceramide mass elevation and caused a further decrease in total long-chain ceramides. Thus, this experiment indicates that C_6-ceramide and *threo*-PPMP induce similar elevation of endogenous long-chain ceramides within sympathetic neurons and opens the possibility that it is the long-chain ceramides which have the anti-apoptotic effect in C_6-ceramide-treated neurons. Because Fumonisin B_1 blocked the conversion of C_6-ceramide into long-chain ceramides and eliminated the elevation of endogenous ceramide upon *threo*-PPMP treatment, I used this observation to discriminate the exogenous (short-chain analogue) from the endogenous effects of C_6-ceramide. Hence, I tested whether Fumonisin B_1 could block C_6-ceramide effect on apoptosis. I treated neurons without or with Fumonisin B_1 for 24 h prior to NGF deprivation to ensure the inhibition of ceramide synthase. I added C_6-ceramide at the time of NGF withdrawal and I evaluated apoptosis after 36 h. Fumonisin B_1 only partially abolished the anti-apoptotic effect of C_6-ceramide (Fig. 3-3B). In parallel I tested the effect of Fumonisin B_1 on *threo*-PPMP-induced survival. Different from C_6-ceramide, FB_1 completely reversed the anti-apoptotic effect of *threo*-PPMP. As expected, Fumonisin B_1 itself did not cause any effect on apoptosis. Moreover, as demonstrated before (de Chaves et al.,1997) neither FB_1 nor the combination of Fumonisin B_1 and *threo*-PPMP caused significant cell death in neurons maintained in NGF (Fig. 3-3B) eliminating the possibility of toxic effects of Fumonisin B_1 and PPMP.

3.2.4. C_6-ceramide shares mechanisms of action with NGF

A "commitment point" has been defined as the duration of NGF deprivation at which half of the neurons die despite the addition of a given agent or intervention (Martin et al., 1992). The "commitment point" for a given neuroprotective agent is determined by looking at the time at which 50% of neurons can no longer be rescued from apoptosis by the addition of that particular agent. The commitment point gives information on the rate-limiting step inhibited by the neuroprotective agent (Deshmukh et al., 1996). To determine the time point at which ceramide acts to block apoptosis, I deprived neurons of NGF and gave them C_6-ceramide after various times. Following a rescue period of 48 h, apoptosis was evaluated. For comparison, some cultures were rescued by addition of NGF or the protein synthesis inhibitor cycloheximide, a known blocker of sympathetic neurons apoptosis (Deckwerth and Johnson, 1993). C_6-ceramide and NGF exhibited time courses of rescue that were virtually identical, with 50% of the neurons rescued after 24±2 h (Fig. 3-4). In agreement with previous observations (Deckwerth and Johnson, 1993), the time course of rescue with cycloheximide preceded the time course of rescue with NGF (commitment point ~18 h). These data indicate that ceramide acts late in inhibiting the apoptotic pathway and that the rate-limiting process affected by ceramide may be identical to that affected by NGF.

3.2.5. C_6-ceramide activates TrkA and downstream signaling pathways

I considered the possibility that C_6-ceramide was activating survival pathways similar to those activated by NGF. Target-derived NGF mediates its biological effects by binding to and activating TrkA neurotrophin receptors at nerve terminals.

In sympathetic neurons Nair and collaborators interpreted their findings as short-chain ceramide leading to activation of TrkA in the absence of NGF, although they did not examine TrkA activation directly (Nair et al., 2000). In the presence of NGF, C_6-ceramide enhances TrkA phosphorylation (de Chaves et al., 2001) but whether C_6-ceramide is able to activate TrkA in the absence of NGF and as a consequence support neuronal survival has not been previously examined. Therefore, I investigated by immunoblot analysis the presence of activated TrkA (p-TrkA) and activated Akt (pAkt) in NGF-deprived neurons treated with or without C_6-ceramide. C_6-Ceramide induced TrkA and Akt activation when NGF was absent (Fig. 3-5A). The inhibitor K252a blocked ceramide-activated TrkA indicating that ceramide activates TrkA kinase activity (Fig. 3-5A). The concentration of K252a used for these studies were in accordance to the data reported for sympathetic neurons (MacInnis et al. 2003). Moreover, activation of Akt by ceramide occurred downstream of PI3-kinase activation since treatment with the PI3-kinase inhibitor LY294002 blocked ceramide-dependent Akt activation as effectively as NGF-induced Akt phosphorylation (Fig.3-5A). On the other hand, endogenous ceramides accumulated via the treatment with *threo*-PPMP did not induce TrkA activation suggesting that other mechanisms also take place in ceramide-induced survival.

Simultaneously, I examined the activation of MAPK/ERK by C_6-ceramide and found that upon ceramide treatment in the absence of NGF no significant activation of ERK 1/2 occurred (Fig. 3-5B). In addition, I show that treatment with FB1, that blocked the conversion of short-chain into long-chain ceramides, did not affect ceramide-induced TrkA activation (Fig. 3-5C) indicating that TrkA activation was due to C_6-ceramide and not to the endogenous-long-chain ceramides.

I next tested whether activation of PI3-kinase/Akt was necessary for the anti-apoptotic effect of ceramide. Since LY294002 completely abolished Akt activation I examined apoptosis in neurons deprived of NGF but given C_6-ceramide in the presence or absence of LY294002. The effectiveness of LY294002 was verified in each experiment by complete inhibition of Akt phosphorylation (Fig. 3-5A). I found that in the presence of LY294002, C_6-ceramide was unable to protect from apoptosis (Fig. 3-5D). This experiment indicates that activation of PI3-kinase is essential for C_6-ceramide-induced survival and suggests that Akt is not a direct target for ceramide but ceramide acts upstream or at the level of PI3-kinase. In agreement with previous reports (Kuruvilla et al., 2000), addition of LY294002 only caused modest apoptosis (~30%) after 36 h in NGF-maintained neurons.

In order to establish if the ceramide effect depends on TrkA phosphorylation I tested the effect of the TrkA kinase inhibitor K252a on neuronal survival. Surprisingly, I found little apoptosis (≤25%) in neurons provided 50 ng/ml NGF and 200 nM K252a (MacInnis et al., 2003) and K252a did not significantly affect the anti-apoptotic effect of ceramide (Fig. 3-5D) although it completely inhibited TrkA phosphorylation induced by ceramide or NGF (Fig. 3-5A). I tested different batches and different concentrations of K252a and consistently found small effects on survival. Similar observations on the effect of K252a on neuronal survival were recently made in Dr. Campenot's laboratory. Under the experimental conditions that I used, I found residual phosphorylation of Akt in neurons treated with NGF and K252a which could explain the apoptosis observed.

Similarly, in neurons treated with ceramide and K252a in the absence of NGF, Akt phosphorylation was not abolished.

To corroborate that ceramide was triggering Akt activation I examined in situ activation of Akt by immunocytochemistry (Fig. 3-6A and B). In parallel, activation of caspase-3 was evaluated. I found that in the absence of NGF only neuronal cultures treated with C_6-ceramide alone displayed a number of neurons immunoreactive to pAkt similar to NGF-maintained cultures (compare (a, c) with (b, d, e) in Fig. 3-6A). Addition of LY294002 to C_6-ceramide-treated cultures caused a dramatic decrease in the number of pAkt positive cells and a concomitant elevation in caspase-3 positive cells as well as neurons with fragmented nuclei (Fig. 3-6A d). Interestingly, neurons given NGF with LY294002 remained mostly alive, as judged by the absence of nuclear fragmentation or immunoreactivity to active caspase-3; however these neurons did not show immunoreactivity to pAkt (Fig. 3-6A e). Quantitative data show a direct correlation of percentage of apoptotic nuclei with percentage of neurons immunoreactive to active caspase-3 in every treatment and an indirect correlation with percentage of neurons immunoreactive for pAkt in all groups with exception of cultures kept in NGF with LY294002 (Fig. 3-6B).

Altogether, the experiments presented in this section indicate that C_6-ceramide exhibit neurotrophin-like, neuronal survival activity. All these data suggest that C_6-ceramide activates non-ligand bound surface TrkA receptor and activates the PI3-kinase/Akt pathway.

3.2.6. Ceramide supports survival from distal axons

In order to test if ceramide was able to activate survival mechanisms when added exclusively to distal axons, I first confirmed that C_6-ceramide provided to the neurons exclusively to distal axons was not increasing ceramide levels in cell bodies or that C_6-ceramide given to the distal axons causes increase in long-chain ceramides in cell bodies. For the experiment I cultured sympathetic neurons in compartmented dishes in which cell bodies and proximal axons (hereafter referred as the cell body compartment) are separated from distal axons and can be treated independently (Campenot, 1982; de Chaves et al., 1997). At 8 days in culture, half of the neurons were given C_6-ceramide in distal axons exclusively. After 24 h, the mass content of C_6-ceramide and long-chain ceramides was analyzed in the cell body-containing compartment and in distal axons separately. A typical experiment is presented in Fig. 3-7A. The band corresponding to C_6-ceramide was only present in the distal axons compartment of neurons treated with the short-chain ceramide analogue but not in the corresponding cell body-containing compartment. Untreated neurons did not contain C_6-ceramide in either compartment. As was the case of mass cultures, treatment with C_6-ceramide in distal axons caused an increase in long-chain ceramides levels that was restricted to the side compartment (Fig. 3-7A and B). These observations indicate that the use of the three-compartment culture system is appropriate and that the effects obtained with ceramide in distal axons should be attributed to local elevation of ceramide in distal axons rather than transport of ceramide to the cell body-containing compartment.

I next tested the ability of C_6-ceramide given to each compartment to support survival. Neurons cultured in compartmented dishes were deprived of NGF by giving α-NGF in all three compartments (maximum apoptosis). Other sets of cultures were given C_6-ceramide together with α-NGF in: distal axons exclusively, cell body-containing compartment only or both cell body-containing compartment and distal axons. Control neurons were cultured with NGF in distal axons and α-NGF in the cell body-containing compartment (no apoptosis). I evaluated apoptosis after 36 h. As shown by others (Kuruvilla et al., 2000; MacInnis and Campenot, 2002), NGF added exclusively to distal axons was sufficient to support the survival of most neurons as demonstrated by the absence of nuclear fragmentation or immunoreactivity to active caspase-3 (NGF group in Fig. 3-8A and B). Deprivation of NGF caused massive nuclear fragmentation with the majority of the neurons showing immunoreactivity for active caspase-3 (no-NGF). Cultures given C_6-ceramide together with α-NGF in all three compartments displayed few caspase-3 positive neurons and very modest nuclear fragmentation (CerCBDAX). These cultures were equivalent to the mass cultures presented in Fig. 3-1. Interestingly, C_6-ceramide provided only to the cell bodies and proximal neurites (C_6CerCB) or only to the distal axons (C_6CerDAX) was able to sustain neuronal survival. The inactive isomer C_6DHCer was unable to support survival even when added to all three compartments (DHCer CBDAX). Quantification of apoptosis in each treatment group is presented in Fig. 3-8B. Similar effects were observed when *threo*-PPMP was used to elevate ceramide in compartmented cultures. Cultures given *threo*-PPMP together with α-NGF in all three compartments (t-PPMPCBDAX); only to the cell bodies and proximal neurites (t-PPMPCB) or only to the distal axons (t-PPMPDAX) displayed few caspase-3 positive neurons and very modest nuclear fragmentation (Fig. 3-8C and D). On the other hand, the inactive isomer *erythro*-PPMP even if given to all three compartments was unable to protect neurons against NGF deprivation induced apoptosis. Addition of C_6-ceramide exclusively to distal axons was as effective as addition of C_6-ceramide to the cell body-containing compartment or to all three compartments in the inhibition of apoptosis. Furthermore, in agreement with our findings in mass cultures, addition of the PI3-kinase inhibitor LY294002 to the same compartment to which C_6-ceramide was provided, completely blocked ceramide-supported survival (Fig. 3-9). The dramatic inhibition of ceramide anti-apoptotic effect by LY294002 in compartmented cultures represents additional evidence for a local effect of ceramide at the distal axons since the inhibitor LY294002 was present exclusively in the compartment in which ceramide was added. If sufficient ceramide would be transported from distal axons to the cell body-containing compartment, LY294002 would have not been effective if present only in DAX. The suitability in the use of LY294004 in compartmented cultures to inhibit PI3-kinase locally has been previously demonstrated (Kuruvilla et al., 2000; MacInnis and Campenot, 2002). As indicated for the case of mass cultures, neurons maintained in compartmented cultures with C_6-ceramide for 36 h were entirely viable presenting more than 90% survival after up to 14 days upon NGF replenishment (data not shown).

Taken together, the experiments presented in this section indicate that addition of C_6-ceramide exclusively to distal axons is sufficient to block apoptosis of sympathetic neurons and suggest that in response to ceramide and in the absence of NGF a signal is generated in distal axons and delivered to cell bodies to support neuronal survival.

3.2.7. p75^{NTR} and SM cycle in sympathetic neurons

In view of the impressive anti-apoptotic effects of ceramide in sympathetic neurons it was important to identify the physiologically relevant agonist for the generation of ceramide in this neuronal cell type. Binding of neurotrophins to p75^{NTR} leads to accumulation of ceramide from SM breakdown (Dobrowsky et al., 1994), however the simultaneous presence of phosphorylated TrkA blocks activation of the SM cycle (Bilderback et al., 2001; Dobrowsky et al.,1995). To investigate whether activation of the SM cycle occurs in sympathetic neurons in response to p75^{NTR} signaling, I deprived neurons of NGF and challenged them with a neurotrophin that binds only to p75^{NTR}, e.g. BDNF. Thus, NGF-deprived neurons were given BDNF in medium containing α-NGF. Alternatively, NGF was used under conditions in which phosphorylation of TrkA was inhibited; that is, some cultures were incubated with K252a for 1 h followed by the addition of medium containing NGF and K252a. Control cultures received NGF. I examined SM hydrolysis and ceramide accumulation. Treatment with BDNF or NGF in the presence of K252a caused a decrease in the levels of SM of ~25% which was evident at 15 min (Fig. 3-10A). Further hydrolysis of SM, however did not occur at later times. I found that concomitantly with the decrease in SM there was an increase in the total mass of ceramides in neurons given BDNF or NGF with K252a but not in those neurons given NGF alone (Fig. 3-10B). The elevation of ceramides was, however, transient. These experiments demonstrate, for the first time that conversion of SM into ceramide occurs in sympathetic neurons downstream of p75^{NTR} activation.

Activation of p75^{NTR} with ceramide generation has been mostly linked to the induction of apoptosis (Brann et al., 2002 ;Casaccia-Bonnefil et al., 1996). Moreover, activation of p75^{NTR} directly signals apoptosis in a Trk-independent fashion in sympathetic neurons (Majdan et al., 2001). Since I demonstrated that ceramide does not induce apoptosis but protects from apoptosis in sympathetic neurons, I examined the role of the ceramide pool generated by p75^{NTR} activation, on neuronal survival. If the amount of ceramide produced by BDNF binding to p75^{NTR} would be sufficient, neurons provided with BDNF should survive. The experiments shown here demonstrated however, that

BDNF was not able to support the survival of sympathetic neurons deprived of NGF (Fig. 3-11A). Moreover, addition of BDNF to KCl-maintained, NGF-deprived neurons caused a modest but consistent decrease in survival (Fig. 3-11A), which is in agreement with previous findings (Bamji et al., 1998). Hence, these results suggest that ceramide accumulated from SM hydrolysis via $p75^{NTR}$ activation is not involved in death/survival regulation.

As indicated above, ceramide needs to be present during NGF deprivation to actively block apoptosis. Pretreatment with C_6-ceramide prior to NGF deprivation or treatment with C_6-ceramide for short periods (up to 6 h) after NGF withdrawal is not sufficient to stop neuronal death (data not shown). Therefore, the lack of inhibition of apoptosis by p75-generated ceramide could be explained by the rapid return of ceramide mass to basal levels. A possible explanation for the lack of sustained SM hydrolysis (and ceramide production) was that the level of $p75^{NTR}$ was affected by NGF deprivation. In fact, Miller and collaborators reported the regulation of $p75^{NTR}$ gene expression by NGF in sympathetic neurons (Miller et al., 1991). I therefore looked at the expression of neurotrophin receptors in neurons cultured for 24 h in medium containing NGF or BDNF in α-NGF or 50 mM KCl in α-NGF. Immunoblot analysis indicated that the expression of $p75^{NTR}$ decreased dramatically in cultures maintained with BDNF or KCl in the absence of NGF (Fig. 3-11B). The expression of TrkA, however, was not affected. To further investigate the time course of down-regulation of $p75^{NTR}$ expression, neurons were deprived of NGF for different times up to 36 h and the levels of $p75^{NTR}$ expression were compared to neurons that have been kept in NGF throughout the experiment (NGF) and neurons deprived of NGF for 6 h that had been given back NGF for 15 min (NGF 15 min). Fig. 3-11C shows that a rapid decrease in the expression of $p75^{NTR}$ at 6 h after NGF deprivation occurs. Moreover, I could not detect the presence of $p75^{NTR}$ at 24 and 36 h after NGF deprivation. These data correlate very well with previous reports showing a decrease in $p75^{NTR}$ mRNA after 4–8 h of NGF deprivation (Freeman et al., 1994) or after ~20 h of treatment with LY294002 (Crowder et al., 1998). Together, the results presented here suggest that under conditions of NGF deprivation for periods of time longer than 6 h, levels of $p75^{NTR}$ are too low to induce accumulation of ceramide. This finding adds an additional level of complexity to the cross-talk between TrkA-$p75^{NTR}$ and ceramide generation in sympathetic neurons which is the down-regulation of p75 that occurs upon NGF deprivation. Importantly, all these data indicate that ceramide cannot be considered a second messenger for long-term responses mediated by $p75^{NTR}$ in sympathetic neurons.

3.3. Discussion

I showed that endogenous as well as exogenous ceramides support survival of sympathetic neurons. The work presented here has important implications for the field of ceramide signaling in neurons. While endogenous pools of ceramides may mediate the response to a still undetermined extracellular agonist, the value of the findings presented here resides in the discovery that, in the absence of NGF, C_6-ceramide activates TrkA and downstream pathways leading to neuronal survival. More importantly, C_6-ceramide is able to support survival from distal axons, without being transported to cell bodies.

In addition, the present work indicates clear differences between ceramide actions and signaling in sympathetic neurons and PC12 cells. Since PC12 cells constitute a useful model for neuronal cells, such differences must be taken into consideration. Particularly important is that ceramide selectively activates the PI3-kinase pathway in sympathetic neurons whereas it activates PI3-kinase and MAPK/ERK in PC12 cells (MacPhee and Barker, 1999). Finally, I demonstrate that ceramide generated from $p75^{NTR}$ activation does not play a role in the regulation of neuronal survival in sympathetic neurons.

3.3.1. Characterization of the anti-apoptotic effect of ceramide in sympathetic neurons

Sympathetic neurons from newborn rat superior cervical ganglia are acutely dependent on NGF for survival and undergo programmed cell death upon removal of NGF *in vivo* (Levi-Montalcini and Angeletti, 1968) and *in vitro* (Edwards et al., 1991). The temporal relationships of morphological and biochemical changes that accompany cell death, and the molecular mechanisms essential to death induced by NGF deprivation have been studied extensively and characterized in sympathetic neurons (Deshmukh and Johnson, 1997). Therefore, this system represents a suitable model for the study of apoptosis.

The present study provides evidence, for the first time, that ceramide generated from the "de novo" pathway (*threo*-PPMP treatment) is as effective as C_6-ceramide in inhibiting apoptosis. The effect of C_6-ceramide or *threo*-PPMP was not restricted to block the signs of apoptosis but to keep the neurons viable and responsive to subsequent re-addition of trophic support. I found that more than 90% of the neurons present in the cultures deprived of NGF, but given C_6-ceramide or *threo*-PPMP for 36 h, were able to respond to NGF re-addition and survive for a period of at least 7 days. Conversely, none of the neurons (<1%) deprived of NGF in the absence of ceramide or *threo*-PPMP, could be rescued by NGF. I also showed that treatment of sympathetic neurons with C_6-ceramide causes an increase of endogenous long-chain ceramides. It has been recently demonstrated that generation of long-chain endogenous ceramides in response to C_6-ceramide treatment involves the recycling of the sphingosine backbone of the short-chain ceramide by a process of deacylation (probably catalyzed by a ceramidase) and reacylation (by ceramide synthase) (Ogretmen et al., 2002). In other cell types this increase is due to induction or activation of SMase (Deigner et al., 2001; Jaffrezou et al., 1998). I did not directly exclude this last possibility; however, because the inhibitor Fumonisin B1 abolished the increase in long-chain ceramides that follows C_6-ceramide treatment, the mechanism in sympathetic neurons most likely involves deacylation and reacylation of the sphingosine backbone (Ogretmen et al., 2001). This finding is particularly important because the cellular location of the bioactive pool of ceramide and the potential targets for ceramide action could be different in each case. C_2-ceramide was also converted into long-chain ceramides but the levels of incorporation of ^{3}[H]palmitate into ceramide were lower than those obtained with C_6-ceramide (not shown). This difference was also evident in fibroblasts (Gomez-Munoz., 1995). Fumonisin B_1 reverted the protection of apoptosis by *threo*-PPMP, but only partially blocked C_6-ceramide-induced survival. I therefore concluded that the anti-apoptotic effect of C_6-ceramide is not entirely due to its conversion to long-chain ceramides, but C_6-ceramide itself promotes survival. Consequently, I identified endogenous and exogenous ceramide as true anti-apoptotic mediators in sympathetic neurons. Our findings that neurons are able to convert short-chain ceramide analogues into long-chain ceramides are significant for understanding the possible mechanisms involved in ceramide cellular effects in other neuronal systems.

3.3.2. Mechanism of action of C_6-ceramide

Previous work proposed that ceramide inhibited the death cascade in sympathetic neurons by blocking the oxidative stress induced by NGF deprivation and thus antagonizing the induction of c-jun (Nair et al., 2000). If that were the only mechanism by which ceramide blocks apoptosis, ceramide would be effective only when added early (less than 5–8 h) after NGF withdrawal, at the time of c-jun induction. The data presented here, however, show that ceramide is able to rescue 50% of the neurons when added after 24 h of NGF deprivation, indicating that ceramide may also suppress the apoptotic pathway at a later stage, probably by mechanisms similar to those used by NGF.

Activation of TrkA results in recruitment and phosphorylation of the signaling intermediates Shc and PLC γ. The small GTP-binding protein Ras is activated downstream of Shc and two signaling pathways are the major effectors of activated Ras: the PI3-kinase/Akt pathway and the MEK/MAPK (ERK) pathway. Activation of the PI3-kinase/Akt pathway also occurs by direct interaction of PI3-kinase to TrkA or through adaptor proteins such as Grb2 and/or Gab1 (reviewed in Kaplan and Miller, 2000; Sofroniew et al., 2001). Evidence on the requirement of activation of the PI3-kinase/Akt pathway for NGF-induced survival in sympathetic neurons is controversial (Crowder et al., 1998, Philpott et al., 1997; Tsui-Pierchala et al., 2000). However, activation of PI3-kinase/Akt is sufficient to support survival in the absence of NGF (Crowder et al., 1998; Philpott et al., 1997). Here, I present evidence that in the absence of NGF, C_6-ceramide causes phosphorylation of TrkA. Activation of TrkA by ceramide had been previously demonstrated in PC12 cells (MacPhee and Barker, 1999). However, since ceramide leads to apoptosis in PC12 cells (Edsall et al., 2001; Edsall et al., 1997) the relevance for ceramide-induced TrkA in this neuron-like system is unknown. In addition, a very interesting and important difference between Barker's work in PC12 cells and the findings shown here is the signaling pathways activated by ceramide. Treatment of PC12 cells with short-chain ceramide causes activation of PI3-kinase and ERK 1/2 (MacPhee and Barker, 1999). The experiments shown in this thesis, on the other hand, demonstrate selective activation of Akt downstream PI3-kinase without concomitant phosphorylation of ERK 1/2. This lack of MAPK/ERK activation has been previously interpreted as an indication that ceramide does not act via TrkA (Nair et al., 2000). However, the data shown here clearly demonstrate that ceramide activates TrkA but selectively regulates the PI3-kinase pathway. A similar selectivity takes place when sympathetic neurons are maintained with NGF covalently cross-linked to beads (MacInnis and Campenot, 2002). In this work, activation of PI3-kinase without activation of MAPKs has been explained by the lack of TrkA/NGF internalization. In agreement with this notion, ceramide inhibits receptor-mediated endocytosis and NGF uptake in sympathetic neurons (de Chaves et al., 2001). Nevertheless, in PC12 cells activation of MAPK/ERK also occurs in the absence of TrkA internalization (Zhang et al., 2000) which might explain the difference between PC12 and sympathetic neurons. Further examination could provide important information about NGF signaling from the plasma membrane and from endosomes in sympathetic neurons.

The experiments with K252a indicate that ceramide activates TrkA kinase activity. However, I could not definitively rule out that TrkA phosphorylation is essential for ceramide-induced survival since we found that K252a did not cause a substantial inhibition of neuronal survival in NGF- or ceramide-treated neurons even when it abolished TrkA phosphorylation. Similar observations on the effect of K252a on neuronal survival were recently made in Dr. Campenot's laboratory. The data with K252a in this thesis suggest that the lack of a remarkable effect of K252a on survival of NGF-treated neurons might be due to incomplete inhibition of Akt phosphorylation. However, I did not investigate whether this is the result of Akt activation by K252a as recently proposed (Roux et al., 2002).

With respect to ceramide-treated neurons, the survival in the presence of K252a could be explained by a direct activation of the PI3-kinase pathway by ceramide, independent of TrkA activation. Thus, ceramide could activate TrkA at the plasma membrane and concomitantly induce activation of the PI3-kinase pathway. The experiments shown in this study cannot distinguish between these two possibilities. Moreover, ceramide-induced PI3-kinase activation independent of TrkA would explain the lack of activation of the MAPK/ERK pathway.

Alternatively, ceramide could activate the PI3-kinase/Akt pathway through mechanisms dependent on non-receptor tyrosine kinase activity and increased Ras-GTP levels as demonstrated in rat2 fibroblasts (Hanna et al., 1999). Although I have not directly excluded this possibility, the fact that ceramide does not activate the Raf-MAPK pathway which is considered to be the main signaling pathway downstream Ras-GTP, suggests that the activation of the PI3-kinase/Akt pathway might be independent of Ras.

Regulation of Akt by ceramide is cell specific and mechanism-dependent. In erythroleukemic cells, ceramide reduces Akt activity through specific dephosphorylation (Schubert et al., 2000) and in motor neurons ceramide-induced apoptosis is linked to inhibition of Akt (Zhou et al., 1998). I showed that LY294002 inhibits ceramide-induced Akt activation suggesting that Akt is not a direct target of ceramide in sympathetic neurons but ceramide acts upstream PI3-kinase.

The results shown in this thesis do not provide evidence for TrkA being a direct target for ceramide but they support previous suggestions that TrkA activation could result from alterations of plasma membrane properties leading to TrkA-homodimers formation (MacPhee and Barker, 1999). The lack of effect of the inhibitor FB_1 on ceramide-induced TrkA phosphorylation suggests that it is the short-chain analogue (probably by insertion at the plasma membrane) which causes TrkA activation. Moreover treatment with *threo*-PPMP did not cause TrkA activation.

Ceramides at low concentrations exist in membranes as "monomeric", single molecules while above a certain concentration referred as "*critical domain concentration*" they aggregate and segregate into lateral domains within the plane of the lipid bilayer, forming ceramide-rich domains (Holthuis et al., 2001). Interestingly, TrkA presents the unusual property of dimerization and autoactivation when its concentration in the plasma membrane reaches a high enough level (Hempstead et al., 1992; Miller and

Kaplan, 2001). Our laboratory is currently examining the possibility that inclusion of TrkA in "ceramide-rich domains" will create a local higher concentration leading to TrkA dimerization and activation.

3.3.3. Ceramide supports survival from the distal axons

In vivo, neurons are maintained by NGF acting exclusively on distal axons. Since ceramide supports survival of sympathetic neurons, it is conceivable that ceramide activates survival mechanisms when added exclusively to distal axons.

I demonstrated that addition of exogenous ceramide exclusively to the distal axons was sufficient to block the apoptotic program in the nucleus, suggesting that survival signals are generated in the distal axons and transported to cell bodies in the absence of NGF. The effectiveness of LY294002 in annulling the protective effect of C_6-ceramide when added together to distal axons indicate that a survival signal different from ceramide itself is transported to the cell bodies.

Retrograde signaling by neurotrophins after Trk activation is a crucial process that has been extensively and sometimes controversially addressed and discussed (reviewed in Barker et al., 2002; Ginty and Segal, 2002; Heerssen and Segal, 2002; Miller and Kaplan, 2002; Sofroniew et al., 2001). Still, how the neurotrophin signals in distal axons are conveyed to the cell bodies is unknown. Contrary to the generally accepted idea that retrograde transport of NGF is essential for retrograde signaling, MacInnis and Campenot recently demonstrated that mechanisms independent of NGF transport to cell bodies are also able to support neuronal survival (MacInnis and Campenot, 2002). The findings with ceramide in this thesis support this concept and offer a valuable model to decipher the nature of the survival signal retrogradely transported from distal axons to cell bodies in the absence of neurotrophins. Understanding the mechanisms by which ceramide activates TrkA in the absence of its natural ligand NGF and mimics at least some aspects of neurotrophin signaling will give crucial information for the development of small molecules with neurotrophin-like activity. These molecules could be extremely useful in neurological disorders in which treatment with natural neurotrophins have been disappointing (Sofroniew et al., 2001). At present our laboratory is analyzing the nature and the mechanisms of transport of survival signals generated by TrkA activation in the absence of neurotrophins.

3.3.4. Role of p75^{NTR} in the generation of ceramide in sympathetic neurons

Based on the knowledge that neurotrophins activate sphingomyelinases in neuronal and non-neuronal cells (Dobrowsky et al., 1995; Dobrowsky et al., 1994), neurotrophins emerged as good candidates as agonists for ceramide generation in sympathetic neurons. However, ceramide supports survival of sympathetic neurons and activation of p75^{NTR} leads to apoptosis (Majdan et al., 2001). In addition, sympathetic neurons express TrkA and TrkA phosphorylation inhibits p75^{NTR}-dependent sphingomyelin hydrolysis (Bilderback et al., 2001; Dobrowsky et al., 1994). Thus, activation of the sphingomyelin cycle by p75^{NTR} would only take place when TrkA phosphorylation is blocked (NGF+K252a) or NGF is absent and a different neurotrophin (e.g. BDNF) binds to p75^{NTR}. Here, we show that BDNF or NGF in the presence of K252a only cause transient (less than 4–6 h) accumulation of ceramide. Accordingly, sphingomyelin hydrolysis reaches a plateau shortly after BDNF or NGF/K252a treatment started. The temporary elevation of ceramide elicited by BDNF treatment is not sufficient to support the survival of sympathetic neurons in the absence of NGF. Conversely, as was reported before (Bamji et al., 1998), I found modest but consistent decreased survival of neurons maintained in KCl and challenged with BDNF. The limited ceramide generation could be explained by the dramatic decrease in the expression of p75^{NTR} that occurs upon NGF deprivation, which is evident as short as 6 h after NGF withdrawal. Up-regulation of p75^{NTR} by NGF has been previously documented (Miller et al., 1991) and a rapid (~4–8 h) decrease in p75^{NTR} mRNA upon NGF deprivation or treatment of NGF-maintained neurons with the PI3-kinase inhibitor LY294002 has been reported (Crowder et al., 1998; Freeman et al., 1994). The decreased expression of p75^{NTR} protein in NGF-deprived neurons is not the result of the global decrease of RNA and proteins that accompany NGF withdrawal and apoptosis (Estus et al., 1994) but a true reduction in p75^{NTR} levels linked to the absence of NGF, since neurons maintained with 50 mM KCl are viable and healthy but express low levels of p75^{NTR}. Importantly, a direct correlation between the levels of p75^{NTR} expression and the levels of generated ceramide has been reported for neuroblastoma cells (Lievremont et al., 1999) and hippocampal neurons (Brann et al., 2002). In this last model, ceramide-mediated apoptosis occurred only when p75^{NTR} was expressed at high levels. The data shown in this thesis suggest that in sympathetic neurons shortly after NGF deprivation, p75^{NTR} levels decrease significantly and become too low to induce the formation of sufficient ceramide to block apoptosis. These experiments do not exclude the possibility that ceramide generated in this short period could play a role as second messenger in other cellular processes but these data demonstrate that such a pool of ceramide does not support survival. These findings indicate that in addition to the negative cross-talk between TrkA and p75^{NTR} for ceramide generation, in sympathetic neurons, down-regulation of p75^{NTR} expression upon NGF deprivation also limits ceramide formation. Recently, a model has been proposed for sympathetic neurons in which one of the major roles of NGF binding to TrkA is to silence p75^{NTR} -mediated ongoing apoptosis (Majdan et al., 2001). Based on this notion, the down-regulation of p75^{NTR} expression could reflect an additional mechanism of protection against neuronal apoptosis that is triggered in the absence of TrkA activation. Undoubtedly, the physiological/pathological relevance and the mechanism of p75 ^{NTR} down-regulation deserve further study.

3.3.5. Ceramide signaling in neurons: survival or death?

The effects of ceramide in neurons are divergent either killing or protecting neurons. The reasons underlying such differences are difficult to understand in cases in which the mechanism(s) involved in the induction of apoptosis or survival are unknown (Brugg et al., 1996; Ping and Barret, 1998; Irie et al., 1998; Toman et al., 2002). A very limited number of direct targets for ceramide have been identified. The final outcome of ceramide cellular actions will most likely reflect the balance between survival and death signals activated in a neuron-type specific fashion. In motor neurons (Zhou et al., 1998) and cortical neurons (Stoica et al., 2003) ceramide-induced apoptosis involves inactivation of Akt. This process is independent of ceramide-activated protein phosphatase or PI3-kinase in motor neurons (Zhou et al., 1998) but in cortical neurons a phosphatase 2A family member (ceramide-activated protein phosphatase) is activated by ceramide and responsible, in considerable part, for Akt dephosphorylation (Stoica et al., 2003). Conversely, Plummer et al. (2005), in our lab, have recently found that the levels of phosphatase 2A activity are particularly low in sympathetic neurons and we present evidence here that PI3-kinase activity is required for ceramide-induced survival. Comparable differences are evident in ceramide-induced regulation of the transcription factor c-jun in different neuronal types. Recent studies have demonstrated that addition of exogenous C_2-ceramide to cultured cortical neurons induces JNK pathway activation and subsequent c-jun phosphorylation leading to apoptosis (Willaime et al., 2001) but in sympathetic neurons ceramide antagonizes NGF deprivation-induced oxidative stress and c-jun induction (Nair et al., 2000). Finally, the sub-cellular location of ceramide accumulation might influence the activation of different ceramide targets therefore dictating the final effect of ceramide. Accumulation of ceramide downstream sphingomyelinase activation could have a different outcome than the accumulation of ceramide from the "*de novo*" pathway or from exogenous origin. Moreover, the studies shown in this thesis clearly indicate that when using short-chain ceramide analogues it is imperative to investigate whether these analogues are converted to long-chain ceramides in order to fully understand the mechanism involved in ceramide actions.

Lastly, the model of ceramide-induced TrkA activation and survival support will allow us to examine the molecular details of neurotrophin-receptor activation and signaling. The current challenges include to recognize the differences in ceramide and NGF-induced TrkA activation that lead to exclusive activation of PI3K in the case of ceramide; to identify the signal(s) transported from distal axons to cell bodies in the absence of NGF and to discover the targets downstream Akt that mediate ceramide-induced survival.

Taken together the experiments presented in this section indicate that addition of C_6-ceramide exclusively to distal axons is sufficient to block apoptosis of sympathetic neurons and suggest that in response to ceramide and in the absence of NGF a signal is generated in distal axons and delivered to cell bodies to support neuronal survival.

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FIGURES AND LEGENDS

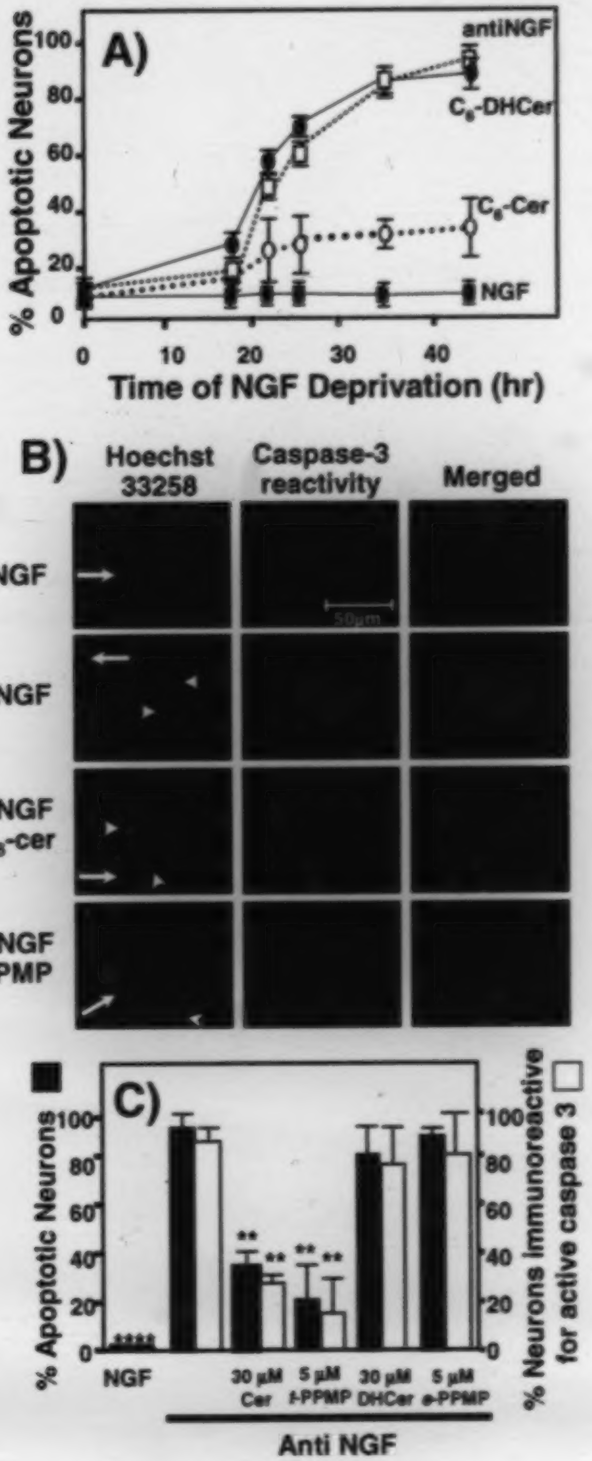
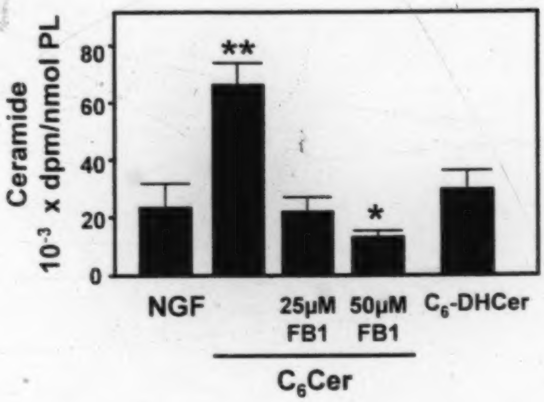
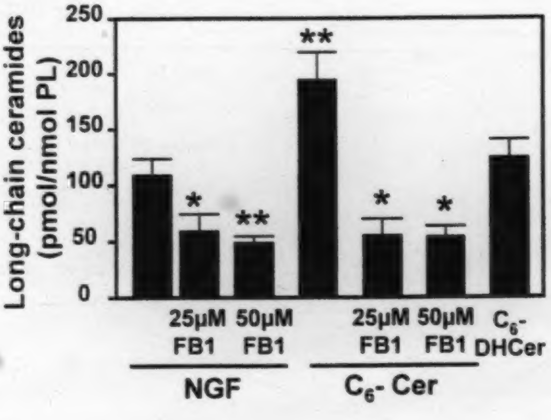

A)
% Apoptotic Neurons
0
20
40
60
80
100
antiNGF
C6-DHCer
C6-Cer
NGF
0
10
20
30
40
Time of NGF Deprivation (hr)
B)
Hoechst 33258
Caspase-3 reactivity
Merged
NGF
no NGF
no NGF + C6-cer
no NGF t-PPMP
50µm
C)
% Apoptotic Neurons
% Neurons Immunoreactive for active caspase 3
0
20
40
60
80
100
**
NGF
30 µM Cer
5 µM t-PPMP
30 µM DHCer
5 µM e-PPMP
Anti NGF

Figure 3-2. C_6-ceramide and *threo*-PPMP induce elevation of endogenous long-chain ceramides. **(A)** Neurons were given medium containing 1 μCi/ml [3H]palmitate. Some cultures were treated with 30 μM C_6-ceramide, 30 μM C_6-ceramide plus FB_1 (25 or 50 μM) or 30 μM C_6-DHCer. After 24 h neurons were harvested, lipids were extracted and analyzed as described under Methods. **(B)** Detection of long-chain endogenous ceramide mass by DAG kinase assay. Cultures were treated as in **(A)** with the exception that neurons received FB_1 were pretreated for 24 h with the inhibitor and that ^{3}[H]palmitate was omitted. The duration of the C_6-ceramide treatment was 24 h. Some cultures maintained in NGF were given FB_1 without C_6-ceramide. Data shown in **(A)** and **(B)** are expressed as means±S.D. of four wells per treatment. Statistically significant differences from cultures given medium lacking C_6-ceramide or FB_1 are indicated by *($p<0.05$) and **($p<0.01$) and were evaluated by the Kruskal-Wallis test with Dunn's Multiple post hoc comparison test. The results shown are representative of at least three independent experiments.

A)


Ceramide
10-3 x dpm/nmol PL
0
20
40
60
80
**
*
NGF
25μM FB1
50μM FB1
C6-DHCer
C6Cer

B)


Long-chain ceramides
(pmol/nmol PL)
0
50
100
150
200
250
*
**
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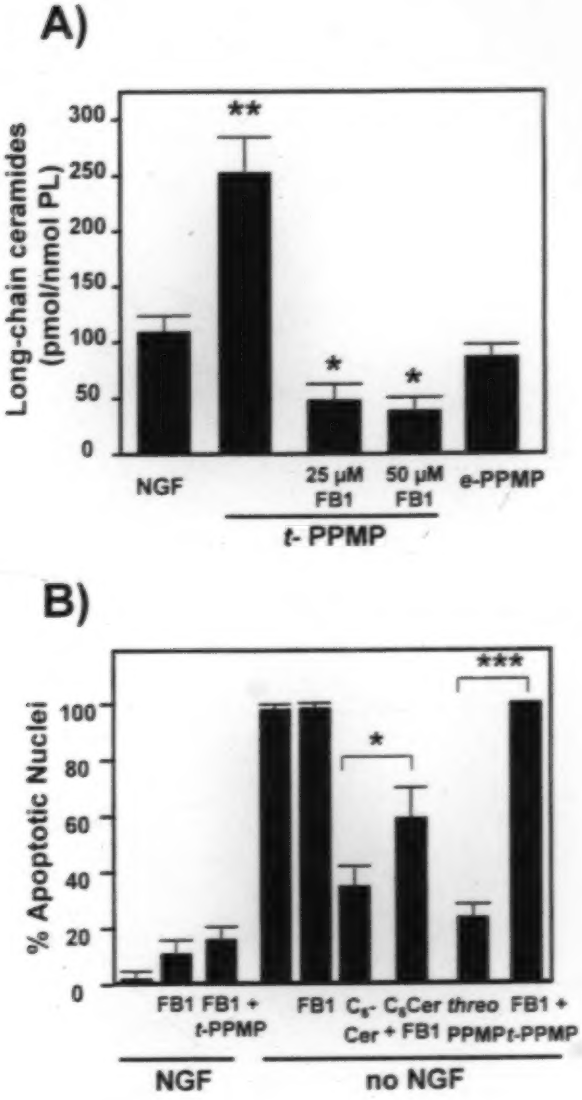
*
*
25μM FB1
50μM FB1
25μM FB1
50μM FB1
C6-DHCer
NGF
C6- Cer

Figure 3-3. The effect of C6-ceramide is due to the short-chain exogenous ceramide and to the long-chain endogenous ceramides. (A) Neurons were treated for 3 d with 5 μM *threo*-PPMP or *erythro*-PPMP, some cultures were given FB_1 for 24 h previous to PPMP treatment and during the 3 d of PPMP treatment. Cellular material was harvested and analyzed as in Fig 2B. Data shown are expressed as means±S.D. of four wells per treatment. Statistically significant differences from cultures given medium lacking PPMP or FB_1 are indicated by *(p<0.05) and ** (p<0.01) and were evaluated by the Kruskal–Wallis test with Dunn's multiple post hoc comparison test. The results shown are representative of at least three independent experiments. **(B)** Effect of FB_1 on C_6-ceramide- and *threo*-PPMP inhibition of apoptosis. Neurons were deprived of NGF and incubated in one of the following: medium containing 8 nM anti-NGF antibody; medium with anti-NGF but containing 30 μM C_6-ceramide; medium with anti-NGF but containing 30 μM C_6-ceramide together with 25 μM FB_1; medium with anti-NGF and 5 μM *threo*-PPMP; medium with anti-NGF together with 5 μM *threo*-PPMP and 25 μM FB_1 and medium with anti-NGF and 25 μM FB_1. Neurons given FB_1 and C_6-ceramide were pretreated with FB_1 for 24 h before NGF deprivation and ceramide addition. Cultures that received FB_1 and *threo*-PPMP were treated with FB_1 for 24 h followed by 3 d in FB_1 and *threo*-PPMP previous to NGF deprivation. Some cultures were given medium containing 50 ng/ml NGF, medium with 50 ng/ml NGF and 25 μM FB_1 or medium with 25 μM FB_1 together with 5 μM *threo*-PPMP. Cultures with NGF and FB_1 received FB_1 for 4 d. Data indicates means±S.D. of 3 experiments performed each with 305 replicates. * indicates statistically significant differences between cultures treated with C_6-ceramide and cultures treated with C_6-ceramide+FB_1 (p<0.05), *** indicates statistically significant differences between cultures treated with *threo*-PPMP and cultures treated with *threo*-PPMP+FB_1 (p<0.001) evaluated by the Kruskal–Wallis test with Dunn's Multiple post hoc comparison test.


A)
Long-chain ceramides (pmol/nmol PL)
0
50
100
150
200
250
300
**
*
*
NGF
25 µM FB1
50 µM FB1
e-PPMP
t- PPMP
B)
% Apoptotic Nuclei
0
20
40
60
80
100
*
***
FB1
FB1 + t-PPMP
FB1
C6-Cer
C6Cer + FB1
threo PPMP
FB1 + t-PPMP
NGF
no NGF

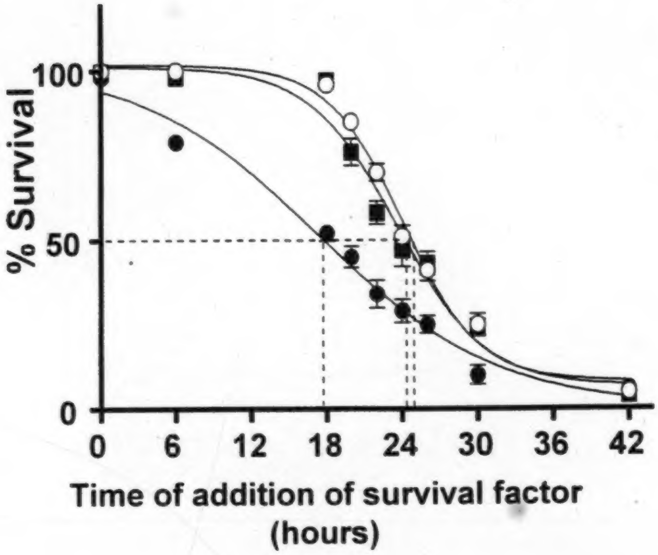


Figure 3-4. Ceramide and NGF have identical time courses of rescue from apoptosis. Comparison of the time courses of rescue with C_6-ceramide, NGF and cycloheximide. Mass cultures of sympathetic neurons were deprived of NGF for the times indicated on the abscissa and then rescued with 30 μM C_6-ceramide (closed squares), 50 ng/ml NGF (open circles) or 1μg/ml cycloheximide (closed circles) for 48 h. The percentage of survival indicates the number of viable neurons relative to the mean number of live neurons when the same neuroprotective agent was added at time 0 h. Values are means±S.D. of five measurements. The experiment was repeated twice with similar results.

Figure 3-5. C_6-Ceramide activates the PI3-kinase/Akt survival pathway. (A) Neurons were deprived of NGF and incubated for 6 h with 8 nM α-NGF, α-NGF and 30 μM C_6-ceramide; α-NGF with 30 μM C_6-ceramide and 200 nM K252a or α-NGF with 30 μM C_6-ceramide and 50 μM LY294002. For the endogenous ceramide group, neurons were treated for 3 d with 5 μM *threo*-PPMP. Some cultures were given NGF with or without K252a or LY294002. Proteins from neuronal cell extracts were separated by SDS-PAGE and identified by immunoblot analysis with antibodies against phosphoTrkA (Y490) and phosphoAkt (S473). After stripping the membranes, anti-total TrkA and anti-total Akt antibodies were used. The figure shows a representative blot. The experiment was repeated four times. Similar results were obtained with treatments ranging between 2 and 19 h of ceramide exposure. **(B)** Ceramide does not induce MAPK/ERK phosphorylation. Neurons were treated and processed as in **(A)**. After protein separation by SDS-PAGE, phosphorylation of MAPK/ERK was examined by immunoblot analysis using an antibody against phospho-MAPK. pTrkA and total TrkA were identified as in **(A)**; tubulin detection was performed using an anti-α-β-tubulin antibody. The experiment was repeated twice with similar results. **(C)** FB_1 does not block ceramide-induced TrkA phosphorylation. Neurons were preincubated with or without 30 μM FB_1 for 48 h. At that time some cultures from each group were deprived of NGF and given 30 μM C_6-ceramide for 18 h. Neurons that received FB_1 during preincubation were also given the inhibitor during the incubation period. Analysis of TrkA phosphorylation was performed as indicated in **(A)**. **(D)** Inhibition of PI3-kinase blocks ceramide anti-apoptotic effect. NGF-deprived neurons were treated with 30 μM C_6-ceramide, 30 μM C_6-ceramide+200 nM K252a or 30 μM C_6-ceramide+50 μM LY294002 for 36 h. Some cultures were given 200 nM K252a or 50 μM LY294002 with NGF. Apoptosis was evaluated by Hoechst 33258 staining. Data indicate means ± S.E.M. of 3 experiments performed in quadruplicates. Statistically significantly differences from cultures given α-NGF are indicated by **(*p*<0.01) and ***(*p*<0.001) and were evaluated by the Kruskal–Wallis test with Dunn's Multiple post hoc comparison test.

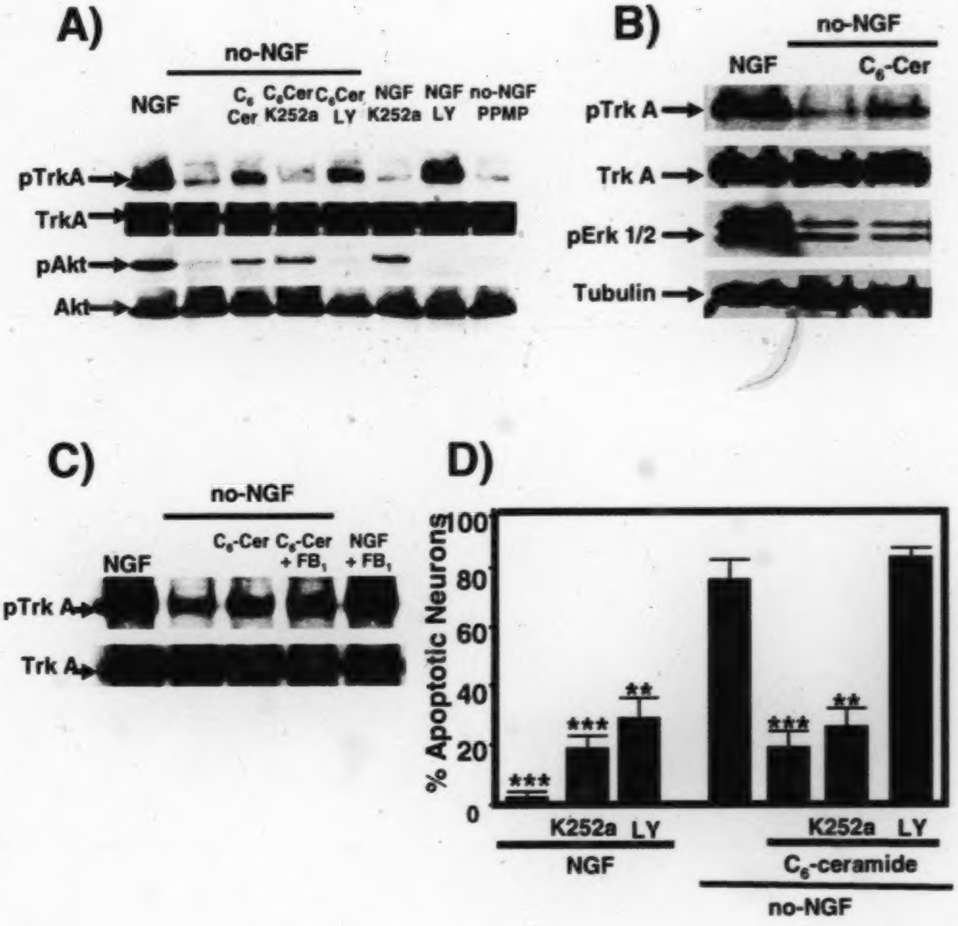

A)
no-NGF
NGF
C_6 Cer
C_6Cer K252a
C_6Cer LY
NGF K252a
NGF LY
no-NGF PPMP
pTrkA
TrkA
pAkt
Akt
B)
no-NGF
NGF
C_6-Cer
pTrk A
Trk A
pErk 1/2
Tubulin
C)
no-NGF
NGF
C_6-Cer
C_6-Cer + FB_1
NGF + FB_1
pTrk A
Trk A
D)
% Apoptotic Neurons
100
80
60
40
20
0
***
***
**
***
**
K252a
LY
NGF
K252a
LY
C_6-ceramide
no-NGF

Figure 3-6. Ceramide increases the number of neurons immunoreactive to activated Akt. Neurons were cultured and treated as in Fig. 3-5A with the exception of K252a. After 36 h, neurons were processed for immunocytochemistry as indicated under Methods. **(A)** *Fluorescence micrographs*: correspond to representative triple merged images of Hoechst 33258 staining (blue), active caspase-3 immunoreactivity (green) and pAkt immunoreactivity (red) for each treatment. NGF (a), 50 ng/ml NGF; no-NGF (b), medium lacking NGF but containing 8 nM α-NGF; no-NGF+Cer (c), medium with no-NGF plus 30 μM C_6-ceramide; no-NGF+Cer+LY (d), medium with α-NGF, 30 μM C_6-ceramide and 50 μM LY294002; NGF+LY (e), 50 ng/ml NGF and 50 μM LY 294002. **(B)** Quantification of the experiment depicted in **(A)**. The percentage of neurons with apoptotic nuclei (Hoechst staining), neurons immunoreactive for active caspase-3 and neurons immunoreactive for active Akt was calculated by counting 3–5 wells per treatment with a total number of neurons per treatment ≥1000. Data are expressed as means±S.D. Symbols on top of bars indicate statistically significant differences from cultures given α-NGF ($p<0.001$) and were evaluated by the Kruskal–Wallis test with Dunn's Multiple post hoc comparison test. * for Akt immunoreactive neurons; ** for active caspase-3 immunoreactive neurons; ‡ for apoptotic neurons (Hoechst 33258 staining). The experiment was repeated two times with similar results.

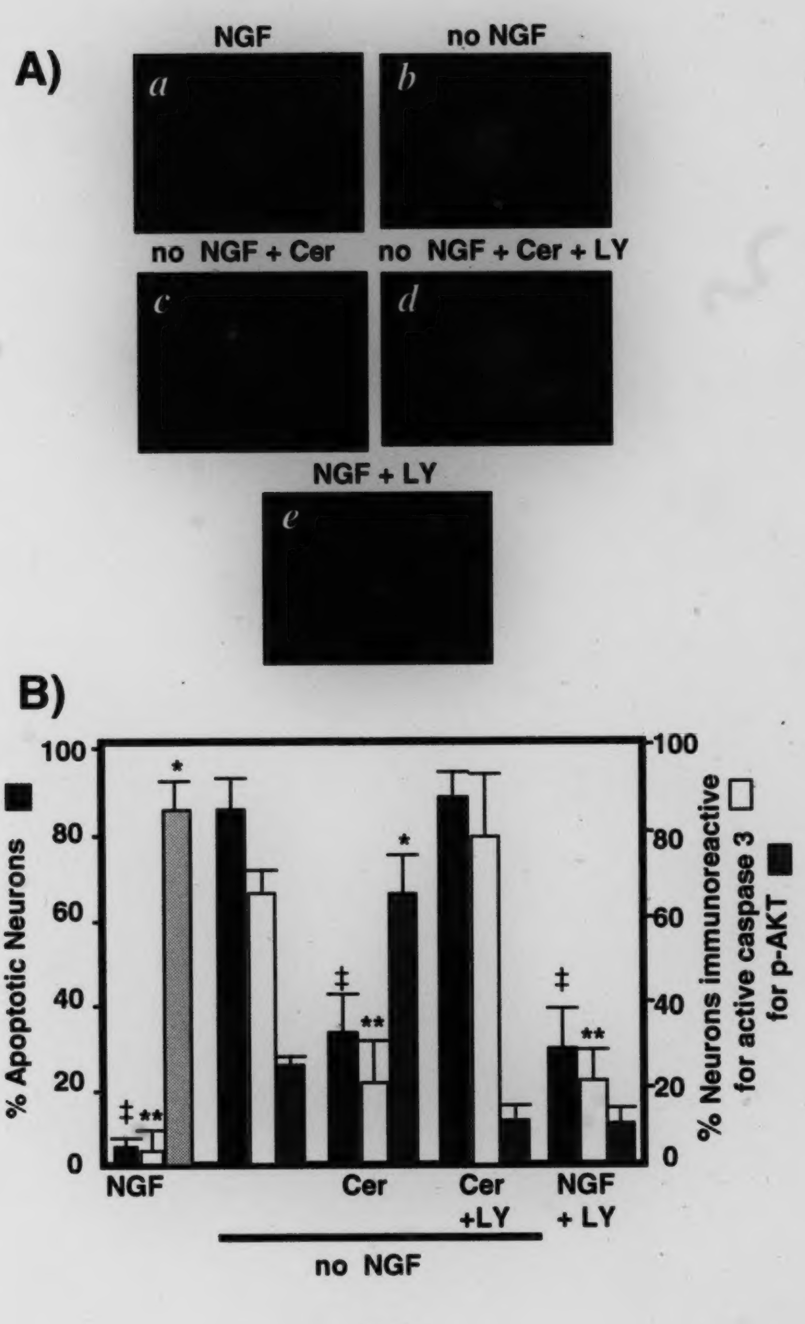
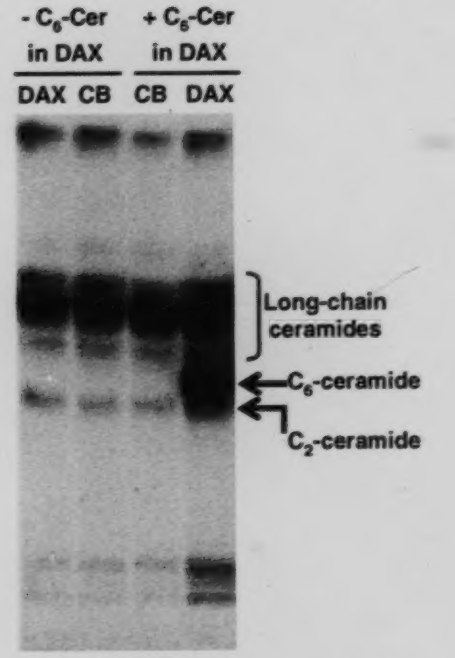

A)
NGF
no NGF
a
b
no NGF + Cer
no NGF + Cer + LY
c
d
NGF + LY
e
B)
% Apoptotic Neurons ■
% Neurons immunoreactive for active caspase 3 □ for p-AKT ■
0
20
40
60
80
100
‡
**
*
NGF
Cer
Cer +LY
NGF + LY
no NGF

Figure 3-7. Addition of C_6-ceramide in distal axons does not cause increase of ceramide in cell bodies/proximal axons. Sympathetic neurons were cultured in three-compartment culture dishes. Half of the cultures were given 30 μM C_6-ceramide in distal axons exclusively while the other half remained untreated. After 24 h cellular material from distal axons (DAX) and cell bodies/proximal axons (CB) was harvested separately and lipids isolated. Levels of ceramide were detected using the DAG-kinase assay. **(A)** Representative TLC plate after DAG-kinase assay showing correspondent bands of ^{32}P-derivative lipids. Note the absence of a band correspondent to C_6-ceramide in all samples with exception of the distal axons to which exogenous ceramides were added. Equal amounts of total lipids from each sample were loaded. **(B)** The amounts of endogenous long-chain ceramides present in each compartment are plotted as percentage of total mass of ceramide present in CB of neurons that did not received C_6-ceramide in DAX. Statistically significant differences from cultures given medium lacking C_6-ceramide are indicated by *($p<0.05$) and were evaluated by the Kruskal–Wallis test with Dunn's Multiple post hoc comparison test.

A)


- C_6-Cer in DAX
+ C_6-Cer in DAX
DAX
CB
CB
DAX
Long-chain ceramides
C_6-ceramide
C_2-ceramide

B)

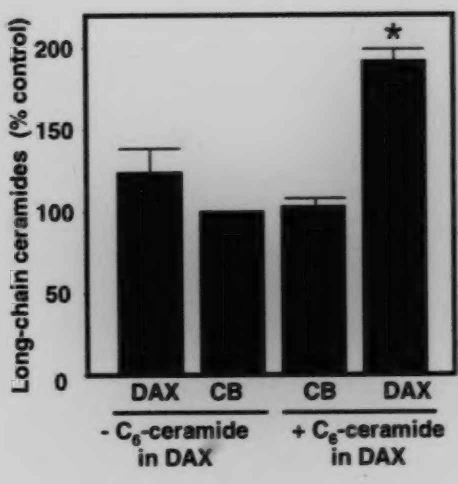
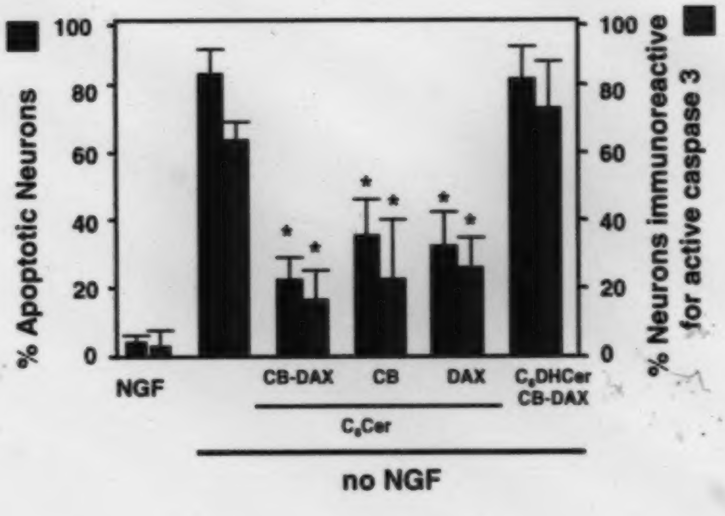

Long-chain ceramides (% control)
200
150
100
50
0
*
DAX
CB
CB
DAX
- C_6-ceramide in DAX
+ C_6-ceramide in DAX

Figure 3-8. Ceramide locally inhibits apoptosis in distal axons. A) and **B)** Neurons were plated in the center compartment of 3-compartment dishes and cultured for 8 days in the presence of 50 ng/ml NGF. Cultures were then deprived of NGF and given one of the follow for 36 h: NGF, 50 ng/ml NGF in the distal axons and 8 nM α-NGF in the cell bodies; no-NGF, 8 nM α-NGF in all three compartments; Cer CBDAX, 8 nM α-NGF and 30 μM C_6-ceramide in all three compartments; Cer DAX, 8 nM anti-NGF in all three compartments and 30 μM C_6-ceramide in distal axons only; Cer CB, 8 nM α-NGF in all three compartments and 30 μM C_6-ceramide in cell bodies and proximal neurites only. **C)** and **D)** Compartmented cultures of neurons were treated with 5 μM *threo-PPMP* in either the cell body-containing compartment (*threo-PPMP* CB), the distal axons containing compartment (*threo-PPMP* DAX) or all three compartments (*threo-PPMP* CBDAX) or with 5 μM *erythro*-PPMP in cell bodies and distal axons (*erythro*-PPMP CBDAX) for 3 days previous to NGF deprivation. Some cultures (NGF, no-NGF) were given medium lacking PPMP. For the NGF deprivation neurons were treated with *threo-PPMP* or *erythro*-PPMP as before but in medium containing 8 nM α-NGF. Apoptotic neurons and neurons immunoreactive for active caspase-3 were identified and counted as in Fig. 1. **A)** and **C)** correspond to representative merged images of Hoechst staining and active caspase-3 immunoreactivity for each treatment. The scale bars indicate 50 μm. In **B)** and **D)** data indicate means ± S.D. of 3-5 experiments performed each with 3-5 replicates. Statistically significantly differences from cultures given α-NGF in all compartments ($p < 0.001$) are indicated by * and were evaluated by the Student's *t* test.

A)

NGF
α-NGF
CerCBDAX
CerDAX
CerCB
DHCerCBDAX

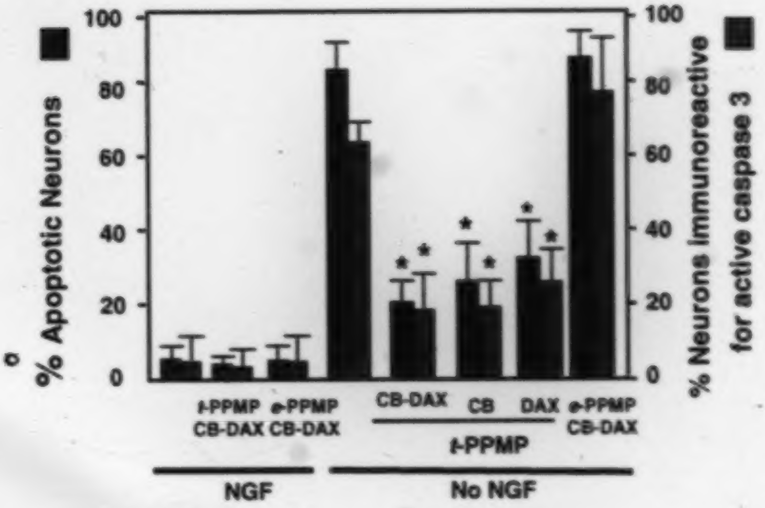
B)


% Apoptotic Neurons
% Neurons immunoreactive for active caspase 3
100
80
60
40
20
0
NGF
CB-DAX
CB
DAX
C6DHCer CB-DAX
C6Cer
no NGF
*

C)

NGF
α–NGF
t-PPMP CBDAX
t-PPMP DAX
t-PPMP CB
e-PPMP CBDAX

D)


% Apoptotic Neurons ■
% Neurons immunoreactive for active caspase 3 ■
100
80
60
40
20
0
*
t-PPMP CB-DAX
e-PPMP CB-DAX
CB-DAX
CB
DAX
e-PPMP CB-DAX
t-PPMP
NGF
No NGF

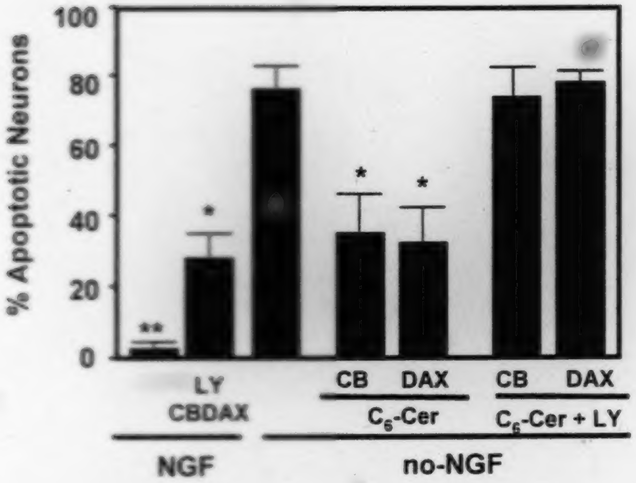


Figure 3-9. Inhibition of PI3-kinase blocks ceramide anti-apoptotic effect. NGF-deprived neurons were treated with 30 μM C_6-ceramide in the CB or DAX as previous. Some cultures received 50 μM LY294002 in the same compartment as C_6-ceramide. Control cultures received 50 ng/ml NGF in DAX. Fifty micromolar LY294002 was added to CB and DAX of certain cultured maintained with NGF. Apoptosis was evaluated by Hoechst 33258 staining.

Figure 3-10. p75^{NTR} induces transient activation of the SM cycle. (A) Hydrolysis of SM. Neurons were metabolically labeled with 1 μCi/ml [*methyl*-^{3}H]choline for 3 days. The label was discontinued and neurons were treated as follows: (i) control group: 50 ng/ml NGF, (ii) preincubated with 200 nM K252a for 1 h prior to treatment with 50 ng/ml NGF and 200 nM K252a (closed squares) and (iii) incubated with 100 ng/ml BDNF in medium containing 8 nM α-NGF (open circles). At the indicated times cellular material was harvested, lipids were extracted and [*methyl*-^{3}H] SM levels were determined as described under Methods. The results are expressed as percentage of SM levels present in control group (NGF-treated neurons). The data shown are means±S.E.M. of five experiments performed in quadruplicates. **(B)** Accumulation of ceramide. Neurons were treated exactly as in (A) but without metabolic labeling. After the appropriate times, cellular material was harvested and ceramide mass was measured by the DAG-kinase assay. The data was normalized to phospholipid content. NGF-treated neurons (open squares); NGF+K252a (closed squares); BDNF-treated neurons (open circles). Values are means±S.E.M. of four experiments performed in triplicates.

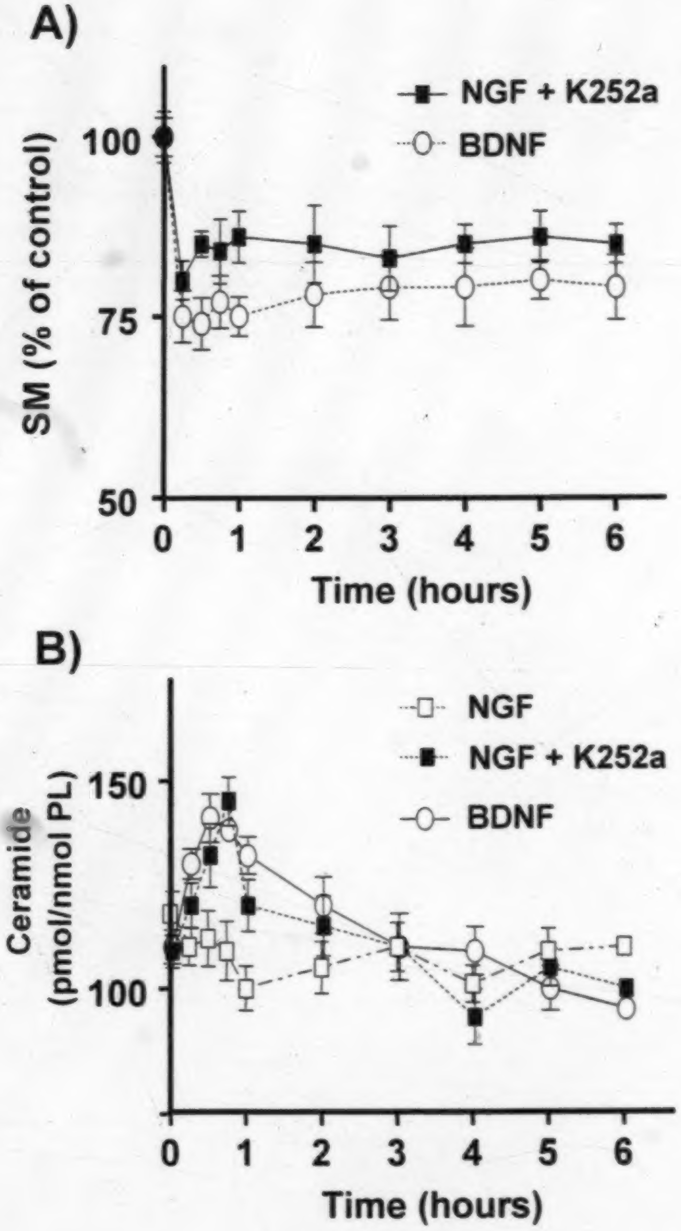
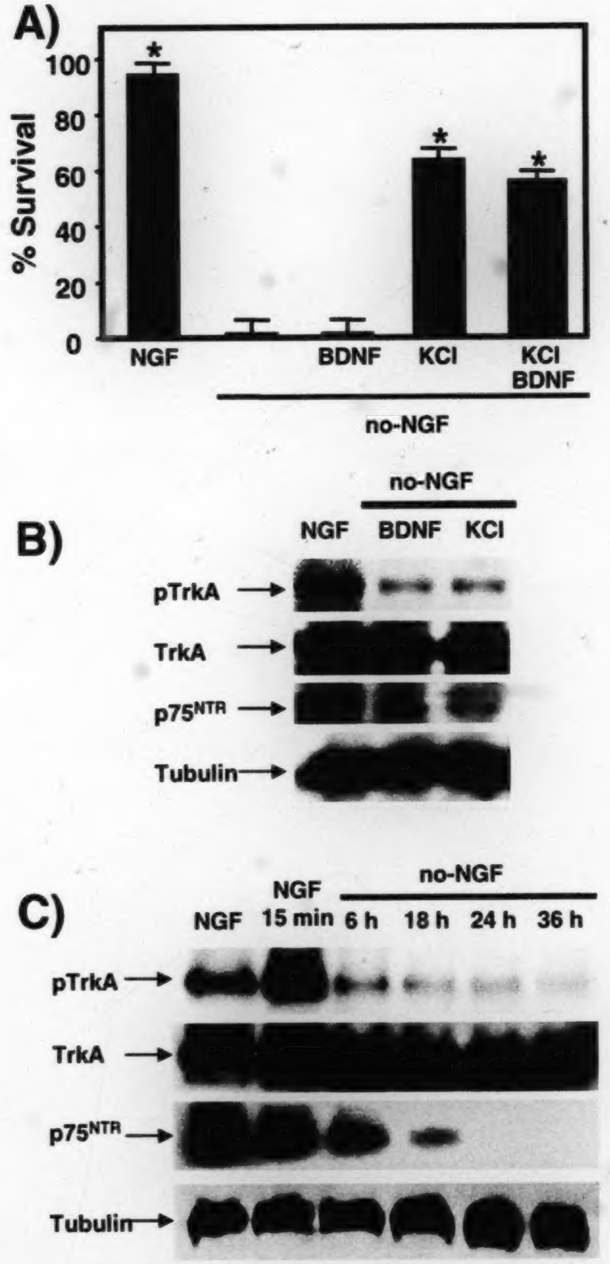

A)
NGF + K252a
BDNF
SM (% of control)
100
75
50
0
1
2
3
4
5
6
Time (hours)
B)
NGF
NGF + K252a
BDNF
Ceramide (pmol/nmol PL)
150
100
0
1
2
3
4
5
6
Time (hours)

Figure 3-11. Activation of p75^{NTR} does not support survival (Regulation of p75^{NTR} by NGF). (A) Sympathetic neurons were washed free of neurotrophins and then cultured for 2 d as follows: NGF, 50 ng/ml NGF; anti-NGF, 8 nM α-NGF; anti-NGF/BDNF, 100 ng/ml BDNF in medium containing α-NGF; KCl, 50 mM KCl in medium containing α-NGF; KCl/BDNF, 50mM KCl and 100ng/ml BDNF in medium containing α-NGF. Neuronal survival was estimated by Hoechst 33258 staining. Each bar represents combined data from four experiments. The number of neurons counted per treatment in each experiment was ≥1000. Data indicate means±S.D. Statistically, significant differences from cultures given α-NGF ($p<0.001$) are indicated by * and were evaluated by the Kruskal–Wallis test with Dunn's Multiple post hoc comparison test. **(B)** Neurons were deprived of NGF and incubated for 24 h with medium containing 8 nM α-NGF and 100ng/ml BDNF or α-NGF and 50 mM KCl. Some cultures were given NGF. Cellular material was harvested and proteins were separated by SDS-PAGE and identified by immunoblot with antibodies against phosphoTrkA (Y490) and p75^{NTR}. After stripping the membranes, anti-total TrkA and anti-α-,β-tubulin antibodies were used. The figure shows a representative blot. The experiment was repeated twice. **(C)** Neurons were deprived of NGF (given medium with α-NGF) for different times up to 36 h. Some cultures were given NGF throughout the experiment (NGF) or for 15 min after 6 h deprivation (NGF 15 min). Cell lysates were prepared and analyzed as in **(B)**. The experiment was repeated three times with similar results.


A)
% Survival
0
20
40
60
80
100
*
*
*
NGF
BDNF
KCl
KCl
BDNF
no-NGF
B)
no-NGF
NGF
BDNF
KCl
pTrkA
TrkA
p75NTR
Tubulin
C)
NGF
NGF
15 min
no-NGF
6 h
18 h
24 h
36 h
pTrkA
TrkA
p75NTR
Tubulin

CHAPTER 4

Aβ-Induced Axonal Degeneration Precedes Nuclear Apoptosis and Is Mediated by a Non-Apoptotic Mechanism that Involves Calpain Activation

Data in this chapter were submitted to Neurobiology of Aging: Song MS and Posse de Chaves EI (2005). Aβ-Induced Axonal Degeneration Precedes Nuclear Apoptosis and Is Mediated by a Non-Apoptotic Mechanism that Involves Calpain Activation

4.1. Introduction

Alzheimer's disease is characterized by neuronal and synaptic loss. The neuropathological hallmarks of Alzheimer's disease are particularly prominent in brain areas such as the parietal and temporal cortices, the hippocampus, the entorhinal cortex, and the amygdale. These regions represent preferential sites for amyloid peptide (Aβ) accumulation and deposition (reviewed in Auld et al., 2002). Brains from Alzheimer's disease patients contain soluble and insoluble assemblies of Aβ, both of which have been suggested to be the cause of dementia (Hardy and Allsop., 1991).

Early evidence from studies in cell culture and *in vivo* pointed at insoluble, fibrillar forms of Aß as the structural form involved in toxicity (Lorenzoet al., 1994; McLean et al., 1999). However, it was later demonstrated that soluble Aβ aggregates (oligomeric Aβ) also induce apoptosis (Pillot et al., 1999) and in some cases are more effective than fibrillar Aβ aggregates (Dahlgren et al., 2002; Lambert et al., 1998; Walsh et al., 2002). Importantly, it has been shown recently that brains of Alzheimer's disease patients exhibit a dramatic increase in soluble oligomeric Aβ (Gong et al., 2003), which is necessary and sufficient to disrupt learning behavior (Cleary et al., 2005).

Several lines of evidence have linked Aβ with neuronal death (reviewed in Dickson, 2004). Aβ causes neuronal death by a mechanism identified as apoptosis, which involves the activation of caspases (Loo et al., 1993; Nakagawa et al., 2000; Jhamandas and MacTavish, 2004; Cribbs et al., 2004). In agreement with this, caspase 3 (Takuma et al., 2004) and caspase 6 (Gong et al., 2003) are activated *in vivo* in Alzheimer's disease brains. In other paradigms Aβ caused neuronal death by mechanisms independent of caspases (Selznick et al., 2000). Independently of the actual molecular mechanism, it is very important to emphasize that the majority of the data available on Aβ-induced neuronal death are the result of studies on cortical or hippocampal neurons (Loo et al., 1993; Estus et al., 1997; Allen et al., 1999) and more rarely on basal forebrain cholinergic neurons (Jhamandas and MacTavish, 2004), which in all cases were exposed to Aß in axons as well as cell bodies. It is still unclear how Aβ activates death mechanisms. Some studies support the notion that a direct effect of Aβ on neurons is required.

Interaction of Aβ with the neurotrophin receptor $p75^{NTR}$ (Kuner et al., 1998), RAGE (Yan et al., 1996) and α-7AChR (Wang et al., 2000) has been linked to Alzheimer's disease. Recently, Jhamandas and MacTavish (2004) demonstrated that Aβ-induced apoptosis of basal forebrain cholinergic neurons is blocked by an amylin receptor antagonist.

Aβ also affects neuronal processes directly inducing a local apoptotic process in neurites (Ivins et al., 1998) and in synaptosomes and dendrites (Mattson et al., 1998) of hippocampal neurons. In addition, recent studies in transgenic mice indicated that the neuritic dystrophy present in Alzheimer's disease models is secondary to Aß and is reversible (Lombardo et al., 2003; Stern et al., 2004; Brendza et al., 2005).

Aβ-induced neuritic pathology might have exceptional significance to basal forebrain cholinergic neurons that differs from other neurons affected in Alzheimer's disease in that their somas are located in nuclei that are physically remote from areas of preferential Aβ accumulation (Jacobs et al., 1992; Amstrong et al., 1994; D'Andrea et al., 2001). Therefore, distal axons of basal forebrain neurons are more likely to be exposed and damaged by Aβ than cell bodies. From the several neuronal populations implicated in Alzheimer's disease, degeneration of basal forebrain cholinergic neurons represents one of the earliest pathological events in Alzheimer's disease, and basal forebrain cholinergic deficits correlate positively with cognitive impairments (reviewed in Auld et al., 2002). A hypothesis of Aβ-induced retrograde degeneration of neurons from the magnocellular nucleus basalis has been proposed (Harkany, 2000). Testing this hypothesis at the molecular levels represents a great challenge due to the particular spatial distribution of these neurons, which cannot be mimicked using conventional culture systems.

Several studies have indicated that Aß induces cholinergic hypofunction in basal forebrain neurons (Auld et al., 2002; Kar et al., 2004). Accordingly, current therapy for Alzheimer's disease focuses in the support of cholinergic function. Ultimately however, therapy should target the prevention of basal forebrain cholinergic neurons death. Significantly, evidence from studies *in vivo* and in animal models supports the view that impaired target-derived trophic support of basal forebrain cholinergic neurons plays an important role in Alzheimer's disease (reviewed in Salehi et al., 2003; Auld et al., 2002).

In the present studies I used a model of neurons cultured in three-compartment dishes in order to exclusively expose axons to Aβ. I aimed to investigate the mechanisms involved in Aß-induced neuronal death in a paradigm relevant to neurons that project to areas of Aß accumulation.

4.2. Results

4.2.1. Aβ-induced neurite degeneration takes place before apoptosis in basal forebrain cholinergic neurons and sympathetic neurons in culture.

The aim of this study was to understand the mechanisms involved in death of neurons exposed to Aβ exclusively in axons termini (*e.g.* basal forebrain neurons). I chose a compartmented culture system (Campenot, 1992), which allows the selective treatment of distal axons with Aβ, and mimics more closely the situation *in vivo.* Cultured forebrain cholinergic neurons do not extend axons sufficiently long enable to cross into side compartments in the Campenot chamber and therefore for the studies in compartmented cultures I used cultured sympathetic neurons of compartmented culture (de Chaves, 1997; Song and Posse de Chaves, 2003). Although sympathetic neurons belong to the peripheral nervous system, they represent a very good model for studying certain aspects related to Alzheimer's disease. Sympathetic neurons share with basal forebrain cholinergic neurons characteristics relevant to Alzheimer's disease development, particularly trophic support and surface receptor expression. As previously discussed, sympathetic neurons depend on the binding of NGF to its receptors TrkA and $p75^{NTR}$ for survival and growth (Kaplan and Miller, 2000). Similarly, basal forebrain neurons express TrkA and p75 (Holtzman et al., 1995; Mufson and Kordower, 1989; Sobreviela et al., 1994) and survival and appropriate function and morphology of adult basal forebrain neurons depends on NGF supply (reviewed in Auld et al., 2002). More importantly, NGF and its receptors have been implicated in the development of Alzheimer's disease (Hock et al., 1998; Capsoni et al., 2000). In addition, as indicated above, the neurotoxic effect of Aβ might be mediated by $p75^{NTR}$ (Kuner et al., 1998).

In order to validate the use of sympathetic neurons for the studies of Aβ-induced neuronal death, I examined whether Aβ would induce death in this neuronal type. I found that treatment of sympathetic neurons in regular cultures (24-well dishes) with $A\beta_{1-42}$ but not with the reverse peptide $A\beta_{42-1}$ for 48h induced apoptosis in a concentration-dependent manner (Fig. 4-1). The concentration-response was very similar to that reported for cultured basal forebrain cholinergic neurons (Jhamandas and MacTavish, 2004), cortical neurons (Mattson et al., 1992), hippocampal neurons (Pike et al., 1993), PC12 cells (Alvarez et al., 1998) and non-neuronal cells (Munoz et al., 2002).

During the course of the above experiments I observed neurite beading and degeneration upon $A\beta_{1-42}$ treatment at a time (18h) when cell bodies looked healthy and did not show signs of apoptosis as demonstrated by the lack of nuclear condensation/fragmentation (Fig. 4-2). The effect was specific to $A\beta_{1-42}$ since the reverse peptide $A\beta_{42-1}$ was unable to induce apoptosis or axonal degeneration. Similar effects were found in basal forebrain cholinergic neurons. These observations suggested that $A\beta_{1-42}$ might activate a destructive program in axons independently of neuronal death as it was previously demonstrated for hippocampal neurons (Ivins et al., 1998). Moreover, it opened the possibility that Aß-induced neuronal apoptosis would be secondary to axonal degeneration.

4.2.2. Neurons exposed to Aβ in distal axons are more susceptible to apoptosis than neurons exposed in cell bodies.

I next investigated the differential susceptibility of distal axons and cell bodies to $A\beta_{1-42}$ in compartmented cultures. I performed a comparative experiment using $A\beta_{1-42}$ in the monomeric (mAβ), oligomeric (oAβ) and fibrillar (fAβ) forms (Dahlgren et al., 2002). The absence of fibrils in the mAß and oAß preparations was confirmed by electron microscopy (data not shown). $A\beta_{1-42}$ (20 μM) was provided to the cell body-containing compartment or to the distal axons-containing compartments separately (Fig. 4-3A) while some cultures remained untreated (control). For comparison in this and subsequent experiments, I included a group of neurons deprived of NGF and provided with anti-NGF antibody (anti-NGF). Apoptosis induced by NGF withdrawal in sympathetic neurons has been characterized extensively (Deshmukh and Johnson, 1997) and has been previously reproduced in our laboratory (Chapter 3. Fig. 3-1). I found that all three forms of Aβ_{1-42} were more effective in inducing apoptosis when applied to distal axons than to the cell body-containing compartment (Fig. 4-3B). Indeed, only oAβ caused significant apoptosis when provided to cell bodies. Although the percentage of apoptosis was consistently higher in neurons treated with oAβ, the differences with mAβ and fAβ were not statistically significant. Nevertheless, in order to standardize Aβ preparations I used oAβ for the rest of the experiments. The results presented in this section further indicate that axons are more susceptible to Aβ than cell bodies and suggest that Aβ-induced nuclear apoptosis is the result of a process initiated in axons.

Using three compartmented cultures I examined axonal degeneration by the decrease in MTT reduction and conversion into a colored formazan salt in the distal axon-containing compartment (MacInnis and Campenot, 2005). This decrease can be quantified by solubilizing the formazan salt and measuring the resultant absorbance at 570 nm. I found remarkable differences between untreated axons and axons treated with Aβ_{1-42}. In untreated neurons, as well as neurons given the inverse peptide Aβ_{42-1}, a profuse bunch of axons, containing abundant colored product was present (Fig. 4-3C). On the other hand, axons treated with Aβ_{1-42} appeared sparse representing much less reduction of MTT. Noticeably, axons exposed to Aβ_{1-42} were morphologically different from axons deprived of NGF in that the retraction and detachment observed in NGF-deprived axons was absent in Aβ_{1-42} -treated neurons.

To examine that axonal degeneration occurs prior to and independently of nuclear apoptosis, I performed a time course experiment in compartmented cultures, treating neurons with oAβ_{1-42} in distal axons exclusively. Axonal viability was quantified in the distal axon-containing compartment and nuclear apoptosis was examined in the cell body-containing compartment of neurons in the same culture dish. Significant axonal degeneration was observed as early as 6h of treatment with Aβ_{1-42}, although nuclear apoptosis occurs later (Fig. 4-4). This finding suggests that axonal degeneration occurs independently of nuclear apoptosis.

4.2.3. Caspases are not involved in axonal degeneration induced by Aβ.

Aβ-induced caspase activation has been reported in synaptosomes (Mattson and Duan, 1999) and indirectly in neurites of cultured hippocampal neurons (Ivins et al., 1998). Whether these neurites are dendrites or axons is unclear. I therefore examined whether caspases were activated locally by exposure of distal axons to $A\beta_{1-42}$. For comparison, I also investigated activation of caspases in neurons deprived of NGF. The presence of activated, cleaved caspases was evaluated by immunoblot analysis. Although procaspase-9 was present in lysates from the cell bodies and distal axons compartments, the 17kD form of cleaved caspase 9 was found exclusively in the cell body-containing compartment of neurons treated with $A\beta_{1-42}$ and in neurons deprived of NGF (Fig. 4-5A left panel). Similarly, the products of caspase-3 cleavage were present in cell bodies of neurons exposed to $A\beta_{1-42}$ or deprived of NGF, but were absent in the distal axons (Fig. 4-5A right panel) indicating that caspase activation does not take place locally in axons of neurons challenged with Aβ or deprived of NGF. In order to confirm that caspase activation was not required for axonal degeneration induced by Aβ, we tested whether BOC-D-fmk, a pharmacological broad-spectrum caspase inhibitor, was able to block Aβ-induced axonal degeneration. BOC-D-fmk (50 μM) was provided to distal axons of some of the cultures treated with Aβ or deprived of NGF. I found that treatment with BOC-D-fmk exclusively in distal axons did not prevent axonal degeneration induced by Aβ or by NGF deprivation (Fig. 4-5B) nor did it prevent nuclear apoptosis (Fig. 4-5C). Conversely, BOC-D-fmk given to the cell bodies completely protected neurons from apoptosis induced by Aβ treatment in distal axons as well as neurons deprived of NGF (Fig. 4-5C). These experiments clearly indicate that Aß-induced axonal degeneration is not mediated by caspase activation. Conversely, caspases are activated in cell bodies in response to local exposure of Aβ in distal axons and local caspase inhibition in cell bodies effectively reduced neuronal apoptosis. The data from NGF-deprived neurons shown here are in agreement with previous reports indicating the lack of caspase involvement in axonal degeneration in this paradigm (Finn et al., 2000; Zhai et al., 2003; MacInnis and Campenot, 2005).

4.2.4. Inhibition of ubiquitin-proteasome system does not prevent axonal degeneration induced by Aβ.

The experiments presented above indicated that Aβ-induced axonal degeneration operates by an apoptosis-independent molecular program similar to that activated in NGF deprivation and/or Wallerian degeneration. Hence, I examined whether inhibition of the ubiquitin-proteasome system would protect axons exposed to Aβ as it protects transected axons or axons deprived of NGF. For the experiments I used MG132, a peptide-based reversible inhibitor of the 26S proteasome with demonstrated activity in axons of sympathetic neurons (Zhai et al., 2003; MacInnis and Campenot, 2005). I first confirmed the effectiveness of MG132 by examining the profile of ubiquitinated proteins by immunoblot analysis. Since proteins targeted for degradation by the proteasome must be first ubiquitinated, I expected to find an increase of ubiquitinated proteins in neurons treated with MG132. I compared the content of ubiquitinated proteins in distal axons of neurons treated without or with MG132. I observed that treatment of neurons with MG132 (5 μM) resulted in an increase of ubiquitinated proteins (Fig. 4-6A), demonstrating that MG132 successfully blocks proteasomal degradation. I next tested whether MG132 was able to prevent Aβ-induced axonal degeneration. I used a treatment design similar to that described for caspase inhibition above. Some neurons were treated with $oA\beta_{1\text{-}42}$ (20 μM) with or without MG132 (5 μM) in distal axons for 36h, at which time I measured axonal viability and apoptosis. As before, some cultures were deprived of NGF and given medium with or without MG132 for comparison. The effect of MG132 by itself was also tested. I found that treatment with MG132 exclusively in distal axons did not prevent axonal degeneration (Fig. 4-6A) or apoptosis (Fig. 4-6B) induced by Aβ. As expected, MG132 treatment caused a reduction in axonal degeneration (Fig. 4-6A) as well as apoptosis (Fig. 4-6B) induced by NGF deprivation. This last result is in agreement with previous evidence from other groups (Zhai et al., 2003; MacInnis and Campenot, 2005). The data presented here not only indicate that the ubiquitin-proteasome system is not involved in Aβ-induced axonal degeneration but imply that the molecular mechanisms of axonal degeneration induced by Aβ and NGF deprivation are different.

4.2.5. Inhibition of calpains has protective effect in Aß-induced axonal degeneration and apoptosis.

I next considered whether calpains, a group of calcium-dependent proteases were involved in Aβ-induced axonal degeneration. In order to examine the relevance of calpain activation I tested if Aβ-induced axonal damage in basal forebrain cholinergic neurons could be prevented by using calpain inhibitors. Among the several calpain inhibitors available I decided to use a peptide derived from the endogenous calpain inhibitor calpastatin (Betts et al., 2003; Eto et al., 1995). The short calpastatin peptide was able to recover neurite viability in Aβ-treated basal forebrain cholinergic neurons as indicated by the improvement of MTT reduction (Fig. 4-7). To further assess the role of local inhibition of calpains in distal axons I tested the ability of calpastatin peptide to prevent axonal degeneration and/or apoptosis in neurons cultured in compartmented dishes and treated with Aβ in the distal axons. As before, I used neurons deprived of NGF for comparison purposes. I found that calpastatin provided to distal axons significantly reduced axonal degeneration (Fig. 4-8A) and nuclear apoptosis (Fig. 4-8B) caused by Aβ but not by NGF deprivation. Similarly, treatment with the calcium chelator EGTA (1 mM) in distal axons was able to protect from Aβ-induced axonal degeneration and neuronal apoptosis (data not shown). Addition of calpastatin to cell bodies of neurons treated with Aβ in the distal axons did not prevent axonal degeneration or nuclear apoptosis (data not shown). Taken together these results indicate that the mechanism activated by Aβ in the induction of axonal degeneration involves local calpain activation in axons and suggest that protection from axonal degeneration is sufficient to prevent Aβ-induced nuclear apoptosis.

4.2.6. Protection of Aβ-induced nuclear apoptosis by NGF.

The data shown here clearly demonstrate that the molecular events involved in Aß-induced axonal degeneration are different than those activated by NGF withdrawal. However, in both circumstances caspase activation occurs at the cell bodies (Fig. 4-5A) and local inhibition of caspases protected from apoptosis (Fig. 4-5C) suggesting that the nuclear apoptotic process might follow common mechanisms. Although these findings suggest that Aβ-treated axons are not deprived of NGF, the possibility still exist that the compromised axons are unable to provide sufficient retrograde neurotrophic support and therefore the cell bodies are deprived of NGF. Indeed, it has been suggested that defective transport of NGF contributes to the degeneration of basal forebrain neurons (Salehi et al., 2003). Noteworthy, under normal culture conditions in three compartment dishes (including all experiments described above) NGF is discontinued from the center compartment at least 48h before the beginning of the experiment. Hence, neuronal survival and axonal growth are supported exclusively from distal axons (Campenot, 1992). If neurons treated with Aβ in distal axons are receiving insufficient amounts of NGF, they should recover if NGF is provided to cell bodies directly. I tested this possibility by adding NGF to the cell body-containing compartment of neurons exposed to Aβ in distal axons. I found that NGF was able to prevent Aß-induced nuclear apoptosis as effectively as it prevented apoptosis caused by NGF deprivation (Fig. 4-9A). I also evaluated the effect of NGF on axonal viability. As expected, addition of NGF exclusively to cell bodies was unable to prevent distal axonal degeneration (Fig. 4-9B). This experiment demonstrates that neuronal apoptosis can be blocked locally at the somas with neurotrophins and suggests that Aβ-induced nuclear apoptosis is the result of a process initiated in distal axons and likely leading to decreased retrograde transport of survival signals.

4.3. Discussion

In this study I show for the first time that exposure of neurons to Aβ exclusively in distal axons causes nuclear apoptosis secondary to axonal degeneration. The studies also show that Aβ-induced axonal degeneration is independent of activation of caspases and the ubiquitin-proteasome system but is reduced by calpastatin, a calpain inhibitor.

4.3.1. Aß-induced axonal degeneration.

Previous studies have proposed axon degeneration and retrograde signal propagation to play a role in Alzheimer's disease pathology (Vickers et al., 1996; Harkany, 2000). Beading and degeneration of neurites have been recognized as early events in neuronal cultures exposed to Aβ (Paradis et al., 1996). In addition, it has been previously demonstrated that Aβ causes direct neurite degeneration in hippocampal neurons cultured in a modified version of the Campenot chamber (Ivins et al., 1998). In the latter work, however, neurites were not identified as dendrites or axons; the activation of neuronal death as a consequence of neurite degeneration was not examined; and the mechanism(s) mediating neurite degeneration are different from the mechanism identified by us (see below).

In our work, neurons are plated in the center compartment of the dish, which is over 1mm wide; therefore, neurites that grow in the side compartments represent distal axons. Thus, the model I used here corresponds better to the situation of basal forebrain neurons *in vivo*. I have demonstrated that exposure of neurons to $A\beta_{1-42}$ exclusively in distal axons leads to decreased axonal viability prior to and independently from apoptosis. Indeed, I found that $A\beta_{1-42}$ is more effective in inducing apoptosis from distal axons than from cell bodies further suggesting that neuronal death is a consequence of a detrimental event that takes place in axons. I have used the reduction of MTT as an indicator of axonal viability. This assay has been previously used to evaluate axonal metabolic status in axons deprived of NGF and axons undergoing Wallerian degeneration (Macinnis and Campenot, 2005). Although Aß could affect MTT reduction by a mechanism that involves increased formazan exocytosis (Liu et al., 1997), it has been indicated that only fibrillar peptides do so (Liu et al., 1998) and therefore this process will unlikely take place under the experimental conditions since I used oAβ. Recently the use of the MTT reduction assay to evaluate Aβinduced death has been questioned (Wogulis et al., 2005). The findings in this study agree with the concern that decreased MTT reduction cannot be inferred as reduced cell viability since extensive axonal degeneration can occur in the absence of neuronal death, particularly during short periods of neuronal exposure to Aß.

4.3.2. Aß-induced axonal degeneration and caspases.

Axon degeneration is an auto-destructive process, comparable to apoptosis, which can be triggered by diverse insults and is mediated by different molecular mechanisms (Coleman and Perry, 2002; Medana and Esiri, 2003; Raff et al., 2002). Understanding the mechanisms involved in axonal degeneration is pivotal for the development of new preventive or therapeutic approaches.

The role of caspases in apoptotic-like events occurring in neurites is controversial. I determined the presence of activated caspases in distal axons directly by immunoblotting and found that although procaspase-3 and procaspase-9 were present in distal axons, local treatment with Aβ or NGF deprivation did not cause their activation. Procaspase-8 was expressed at a much lower level and was not activated (data not shown). This is in agreement with the data obtained in sympathetic neurons globally deprived of NGF in which active caspase-3 detected by immunofluorescence was restricted to cell bodies (Chapter 3. Fig. 3-8). The results presented here suggest that Aβ-induced axonal degeneration is independent of caspase activation. The lack of caspase cleavage products in the axons could be due to a more rapid turn-over in distal axons; however, additional experiments performed at different times intervals from 30 min to 36h failed to detect low molecular weight products at any time (data not shown). Supporting the notion that caspase activation is not involved in Aß-induced axonal degeneration, I demonstrated that the general caspase inhibitor BOC-D-fmk was unable to prevent axonal degeneration or nuclear apoptosis when provided to distal axons exclusively. Since failed neurotrophic support has been suggested as part of the mechanism of neurodegeneration that accompanies Alzheimer's disease (Salehi et al., 2003) (see below), in most of the experiments I also used sympathetic neurons deprived of NGF for comparison. Neuronal apoptosis induced by NGF deprivation has been very well characterized (Deshmukh and Johnson, 1997; Freeman et al., 2004) and axonal degeneration in this paradigm has been examined in the past (Finn et al., 2000; Zhai et al., 2003; MacInnis and Campenot, 2005). The data from NGF-deprived neurons in this study are consistent with earlier reports in dorsal root ganglion neurons in which caspase activation was absent in axons locally deprived of NGF (Finn et al., 2000) and with work from Campenot's lab demonstrating that BOCD-fmk was unable to protect NGF-deprived axons of sympathetic neurons cultured in compartmented dishes (MacInnis and Campenot, 2005). Conversely, Mattson and co-workers reported caspase activation in neurites of hippocampal neurons deprived of trophic support (Mattson et al., 1999) and in synaptosomes exposed to various insults including Aβ (Mattson et al., 1998). More recently a wave of caspase activation moving from the synapses to cell bodies has been detected in olfactory receptor neurons (Cowan et al., 2001). In addition, local treatment of hippocamppal neurites with Aβ and staurosporine caused neurite degeneration and exposure of phosphatidylserine (Ivins et al., 1998). In this work, however, direct demonstration of caspase activation in axons was not provided. Moreover, the effect of Aß on nuclear apoptosis was not examined and, as previously discussed by Raff and collaborators (Finn et al., 2000), the possibility that axonal degeneration was secondary to effects on the neuronal cell bodies was not excluded.

4.3.3. Aß-induced axonal degeneration and the ubiquitin-proteasome system.

Caspase-independent axonal degeneration is characteristic of Wallerian degeneration (Finn et al., 2000; Macinnis and Campenot, 2005). The axonal depletion observed in Alzheimer's disease has been interpreted as Wallerian degeneration secondary to neuronal loss (Medana and Esiri, 2003). Wallerian degeneration involves activation of the ubiquitin-proteasome system at an early stage of the process and accordingly, inhibitors of the ubiquitin-proteasome system prevent axonal damage in transected neurites and in sympathetic neurons deprived of NGF (Zhai et al., 2003; Macinnis and Campenot, 2005). If Aβ-induced axonal degeneration follows a mechanism similar to Wallerian degeneration or NGF deprivation, the ubiquitin proteasome system inhibitor MG132 should prevent axonal damage. The results presented here, however, conclusively demonstrate that axonal degeneration in axons treated with Aβ was not prevented by MG132, while axons of sympathetic neurons deprived of NGF were protected from degeneration by MG132. This result indicates that the ubiquitin-proteasome system is not involved in Aβ-induced axonal degeneration and moreover suggests that the axonal degeneration caused by Aβ is due to a mechanism different than NGF-deprivation. Together with the fact that all the studies on Aβ-induced axonal degeneration presented here have been performed in the presence of NGF in distal axons and that Aβ does not interfere with NGF trophic activity (Troy et al., 2000) the data shown here suggest that delivery of NGF to axons is not affected by Aβ. An additional piece of information supporting this notion comes from the pattern of axonal degeneration observed in Aß-treated axons and NGF-deprived axons; Aß-treated axons do not show the characteristic retraction observed in axons upon NGF withdrawal and they do not detach easily from the substratum as NGF-deprived axons do.

4.3.4. Aß-induced axonal degeneration and calpains.

I next considered the possibility that activation of calpains might mediate Aβ-induced axonal degeneration. Calpains have been implicated in cell aging and neurodegenerative changes and they are activated before the signs of neurodegeneration are apparent in Alzheimer's disease (Nixon, 2000; Nixon, 2003). Indeed, calpains, acting directly or indirectly through other proteolytic pathways and signaling cascades have been suggested to promote the lesions of Alzheimer's disease (Nixon, 2000). I found that the calpain inhibitor calpastatin was able to prevent the loss of axonal viability induced by Aβ in basal forebrain cholinergic neurons. Calpastatin also significantly reduced Aβ-induced axonal degeneration and apoptosis in neurons treated with Aβ in distal axons. Conversely, neurons in which axonal degeneration and apoptosis was induced by NGF deprivation were not protected by calpastatin. These results indicate that calpains are involved in axonal degeneration and apoptosis induced by Aβ and they further suggest that the axonal degeneration seen in neurons treated with Aβ cannot be attributed to local NGF deprivation. Significant for these results, activated forms of calpains have been localized to neurites (Di Rosa et al., 2002; Nixon, 2003). Moreover, $A\beta_{25\text{-}35}$ induced calpain activation in cortical neurons (Li et al., 2003). In addition Jordan and colleagues reported that hippocampal neuronal death induced by $A\beta_{25\text{-}35}$ can be blocked by inhibitors of calpain and caspases (Jordan et al., 1997). Two possible circumstances have been envisioned in the latter work, namely calpains act upstream of caspases or alternatively calpains and caspases act in parallel. The results presented in this study favor the second possibility in which calpains are activated in distal axons and caspases are activated in cell bodies (and possibly dendrites). Calpains are activated by sustained elevation of intracellular Ca^{+2}, an event that takes place in Alzheimer's disease (Mattson and Chan, 2003). Accordingly, I observed that treatment with EGTA protected axons from Aβ-induced degeneration. The mechanism by which Aβ activates calpains to cause axonal degeneration is currently under investigation. Nevertheless, a very important implication of this study is that Aβ-induced neuronal apoptosis can be negatively regulated by reducing axonal degeneration with a calpain inhibitor.

4.3.5. Aß-induced nuclear apoptosis.

To our knowledge, this study is the first direct demonstration that treatment of neurons distally in the axons causes nuclear apoptosis. Aβ has been proposed to cause cell death by activation of apoptotic pathways (Allen et al, 2001; Estus et al., 1997; Nakagawa et al., 2000; Troy et al., 2000). Caspase activation has also been detected in brains of Alzheimer's disease patients (Guo et al., 2004; Rohn et al., 2001). Moreover, Aβ-induced neuronal death is inhibited by the broad-spectrum caspase inhibitor zVAD-fmk (Guo et al., 1997; Jordan et al., 1997; Troy et al., 2000). In agreement, I found activation of caspase-3 and caspase-9 in cell bodies of neurons treated with $A\beta_{1\text{-}42}$ in distal axons and I was able to block Aβ-induced apoptosis by treatment of cell bodies with the caspase inhibitor BOC-D-fmk.

Although mechanistically distinct, the data presented here imply that axonal degeneration and nuclear apoptosis induced by Aβ are coupled such as the latter occurs as a consequence of axon damage. Indeed, I found that $A\beta_{1-42}$ is more effective in inducing apoptosis from distal axons than from cell bodies further suggesting that neuronal death results from a death-related event that takes place in axons. The relevance for such dependence resides in the possibility of protecting neurons from death by blocking axonal damage. The mechanism(s) by which axonal damage leads to nuclear apoptosis require further examination.

Most studies suggest that basal forebrain neurons in Alzheimer's disease are receiving inadequate trophic support or have decreased neurotrophin responsiveness (Salehi et al., 2003; Salehi et al., 2004). NGF is the most important target-derived growth trophic factor for basal forebrain cholinergic neurons; it contributes to the maintenance of the cholinergic phenotype of these neurons and might be critical for certain learning and memory functions (reviewed in Auld et al., 2002). The data shown here indicate that the nuclear apoptosis that follows Aβ-induced axonal degeneration is indistinguishable from apoptosis induced by NGF withdrawal from the distal axons. Moreover, delivery of NGF directly to cell bodies overcomes nuclear apoptosis in neurons treated with Aβ as well as in neurons deprived of NGF. These results suggest that although the provision of NGF to axons might not be altered by Aß, NGF or signaling retrograde transport could be impaired in Aβ-treated neurons. Our lab is currently investigating these possibilities.

In summary, I have demonstrated that exposure of neurons to Aβ exclusively in distal axons activates a destructive program in axons that can be blocked by calpain inhibition. More importantly, these studies support the idea that neuronal nuclear apoptosis is the result of axonal damage and that neurons can be protected if the integrity of the axons is conserved. The studies presented here have important implications for the treatment of Alzheimer's disease. The data shown here support previous suggestions that inhibitors of calpain might be effective therapeutic agents (Jordan et al., 1997); however, they indicate that the targets of calpain inhibitors are localized to axons.

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FIGURES AND LEGENDS

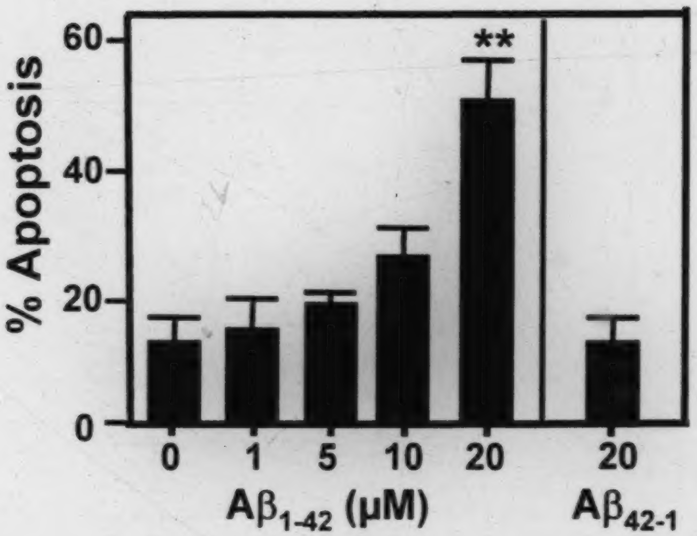
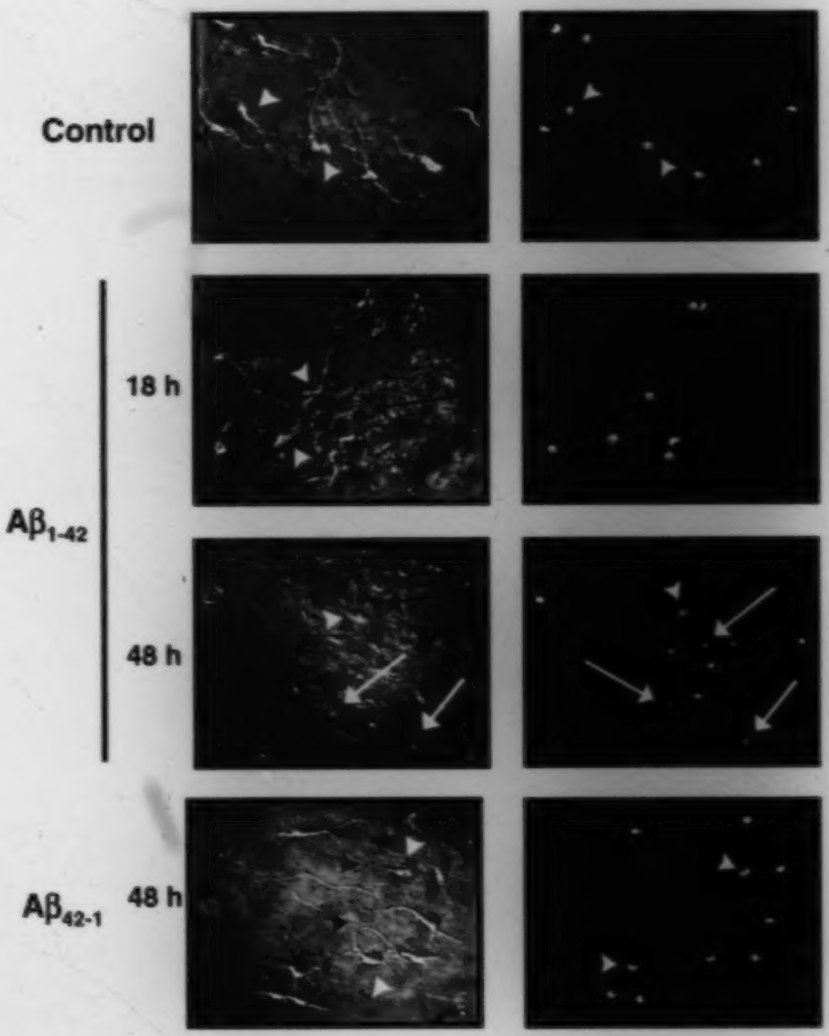


Figure 4-1. Aβ induces apoptosis of cultured sympathetic neurons. Sympathetic neurons cultured in 24-well dishes were incubated with different concentrations of soluble $A\beta_{1\text{-}42}$ or with 20 μM of the reverse peptide $A\beta_{42\text{-}1}$. The percentage of apoptotic nuclei was evaluated by Hoechst 33258 staining. Each treatment group consisted of 3–5 wells and the number of neurons counted per treatment was 500-1000. Data are expressed as means ± S.D. Statistically significant differences from cultures given no Aβ($p < 0.001$) are indicated by ** and were evaluated by the Kruskal–Wallis test with Dunn's multiple post hoc comparison test. The experiment was repeated two times with similar results. Comparable results were obtained when aggregated $A\beta_{1\text{-}42}$ was used (not shown).

Figure 4-2. Aβ induces apoptosis and axonal degeneration in cultured basal forebrain and sympathetic neurons. Basal forebrain neurons (A) and sympathetic neurons (B) were treated without or with 20 μM A β_{1-42} for18h or 48h. Some cultures were given the reverse peptide $Aß_{42-1}$ for 48h. Neurons were examined by phase contrast microscopy. Nuclear apoptosis was evaluated by Hoechst 33258 staining. White arrowheads point to healthy cell bodies (in the phase contrast micrographs) or healthy nuclei (in the fluorescence micrographs), white arrows indicate abnormal cell bodies or apoptotic nuclei respectively. Black arrowheads mark normal neurites while black arrows point to degenerating neurites.



A) Basal Forebrain Neurons

Control
18 h
Aβ1-42
48 h
Aβ42-1
48 h

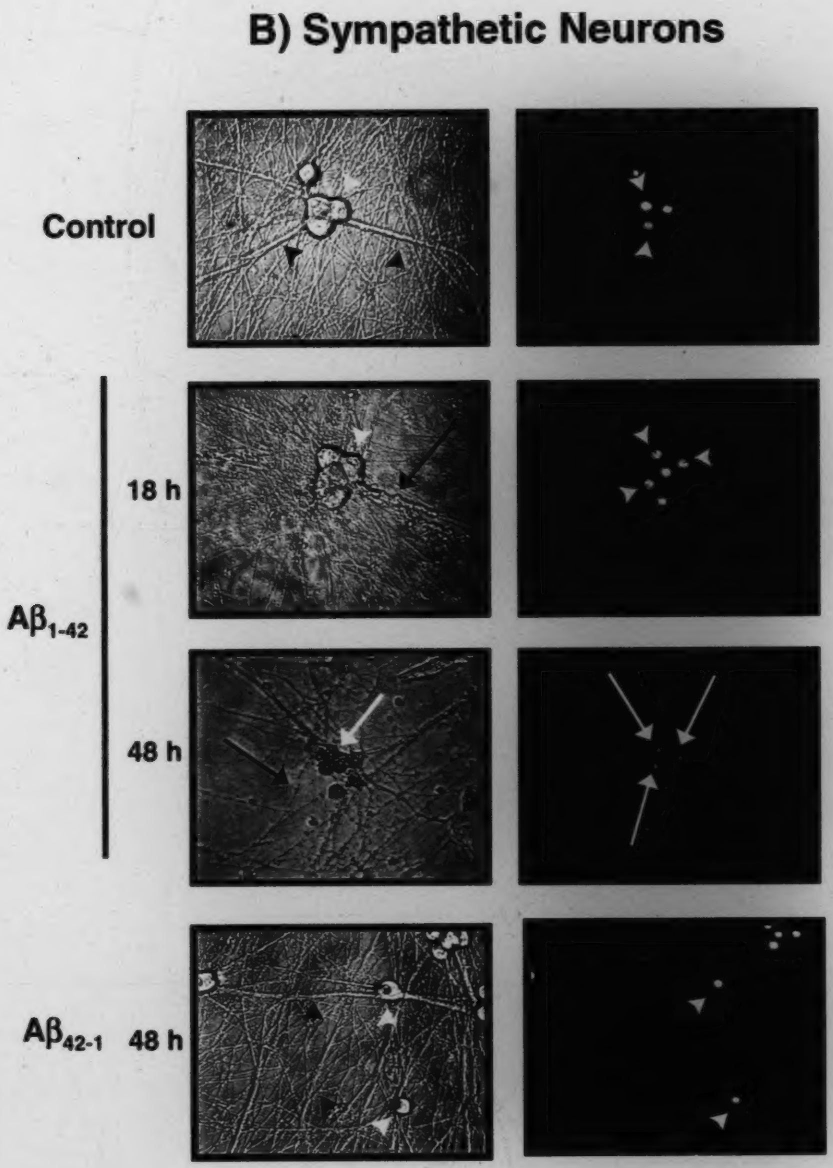
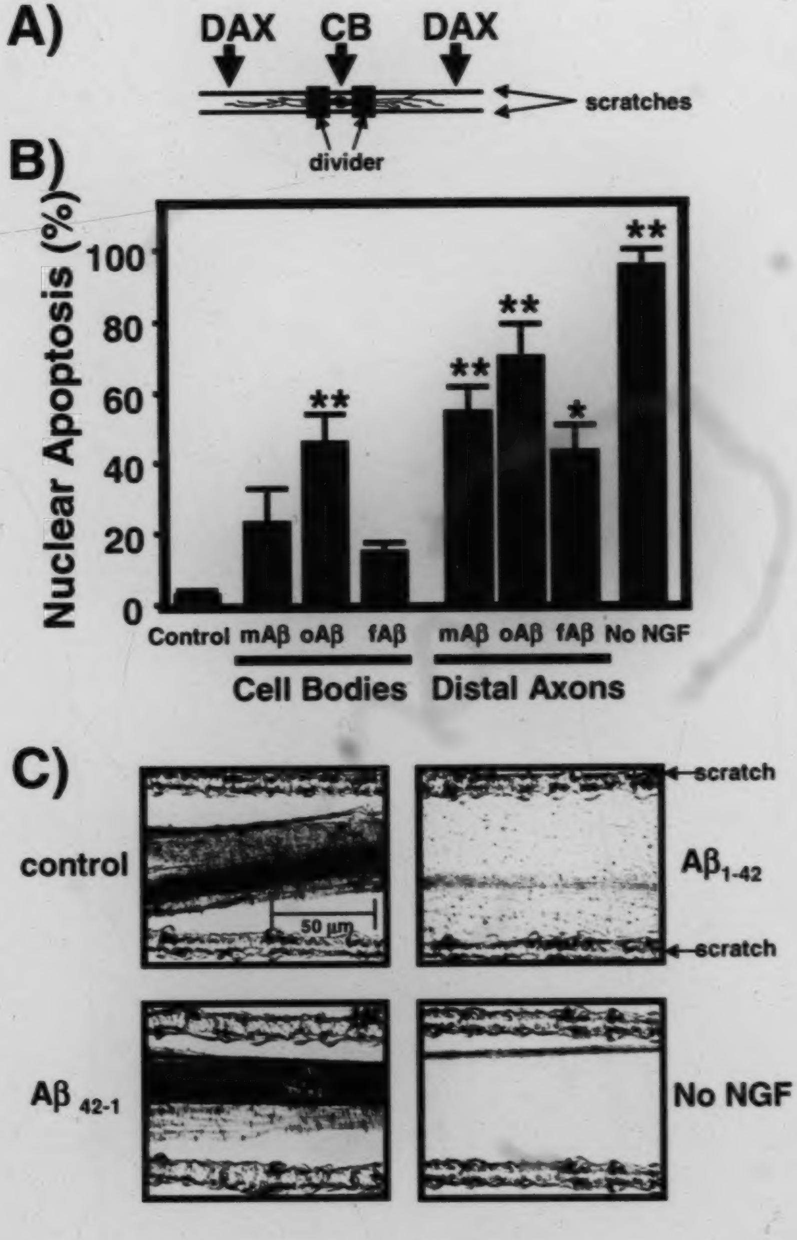

B) Sympathetic Neurons
Control
$A\beta_{1-42}$
18 h
48 h
$A\beta_{42-1}$
48 h

Figure 4-3. Neurons are more susceptible to apoptosis when exposed to Aβ in distal axons. A) Schematic of a single track of a culture in which neurons are plated in the center compartment. CB indicates compartment containing cell bodies and proximal neurites, DAX represents compartment containing distal axons. **B)** Neurons cultured in 3-compartment culture dishes were treated with 20μM of mAβ, oAβ or fAβ in the compartments containing either cell bodies or the distal axons. Neurons received 10ng/ml NGF in distal axons with exception of neurons deprived of NGF in which anti-NGF was provided. All groups received medium with no NGF (8nM anti-NGF) in the CB. Nuclear apoptosis was evaluated by Hoechst staining after 36h of exposure. Data are expressed as means±S.D. Statistically significant differences from untreated cultures evaluated by Kruskal–Wallis test with Dunn's multiple post hoc comparison test are indicated by ** ($p<0.001$) and * ($p<0.05$). The experiment was repeated three times with similar results. **C)** MTT reduction in distal axons of untreated neurons or neurons treated with oA$\beta_{1\text{-}42}$, the inverse peptide A$\beta_{42\text{-}1}$ or deprived of NGF for 18h. Bright field images of part of a single track of the distal axons compartment from a compartment culture are presented.


A)
DAX
CB
DAX
scratches
divider
B)
Nuclear Apoptosis (%)
0
20
40
60
80
100
**
**
**
*
**
Control
mAβ
oAβ
fAβ
mAβ
oAβ
fAβ
No NGF
Cell Bodies
Distal Axons
C)
control
scratch
$A\beta_{1-42}$
scratch
50 µm
$A\beta_{42-1}$
No NGF

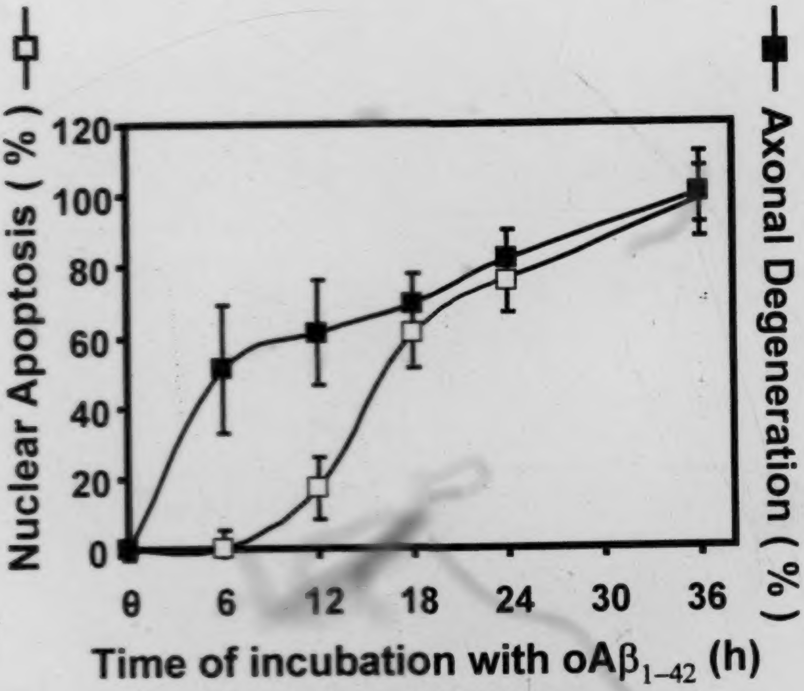


Figure 4-4. **Aβ–induced axonal degeneration precedes nuclear apoptosis.** Neurons cultured in compartmented dishes were treated with oAβ(20μM) in the distal axons compartment for different times ranging from 6 to 36h. At each time nuclear apoptosis (measure by counting the percent of condense/fragmented nuclei) and axonal degeneration (measured by the decrease in absorbance at 570nm) were evaluated in the same dish as indicated in Methods. Values are means ± S.D. and are expressed as percentage of maximum apoptosis or axonal degeneration obtained at 36h. The experiment was repeated two times with similar results.

Figure 4-5. Caspases are not activated in distal axons and do not mediate Aβ-induced axonal degeneration. A) Neurons were treated with oAβ (20 μM) in distal axons or were deprived of NGF. After 48h the cellular material was harvested from the cell body-containing compartment and the distal axons-containing compartments separately. Procaspases-9 and -3 and their correspondent cleavage products were analyzed by SDS-PAGE and immunoblotting as indicated under Materials and Methods. **B)** Neurons cultured in compartmented dishes were treated with oAβ(20 μM) or deprived of NGF (-NGF). Some cultures were given BOC-D-fmk (50μM) in distal axons. None of the neurons received NGF in the cell body-containing compartment. Axonal viability was evaluated by MTT reduction after 36h. Values are means ± S.D. and are expressed as percentage of maximum axonal viability obtained in untreated neurons. There was no significant difference ($p>0.5$) between neurons treated with and without BOC-D-fmk as indicated by the Kruskal–Wallis test. **C)** Neurons were treated as in B). In addition some neurons deprived of NGF or treated with Aß in the axons were given BOC-D-fmk (50μM) in the cell body-containing compartment. Apoptosis was evaluated by Hoechst 33258 staining. Data are expressed as means ± S.D. Statistically significantly differences from cultures given α NGF ($p<0.001$) are indicated by ** and were evaluated by the Kruskal-Wallis test with Dunn's Multiple pot hoc comparison test. The experiment was repeated three times with similar results.

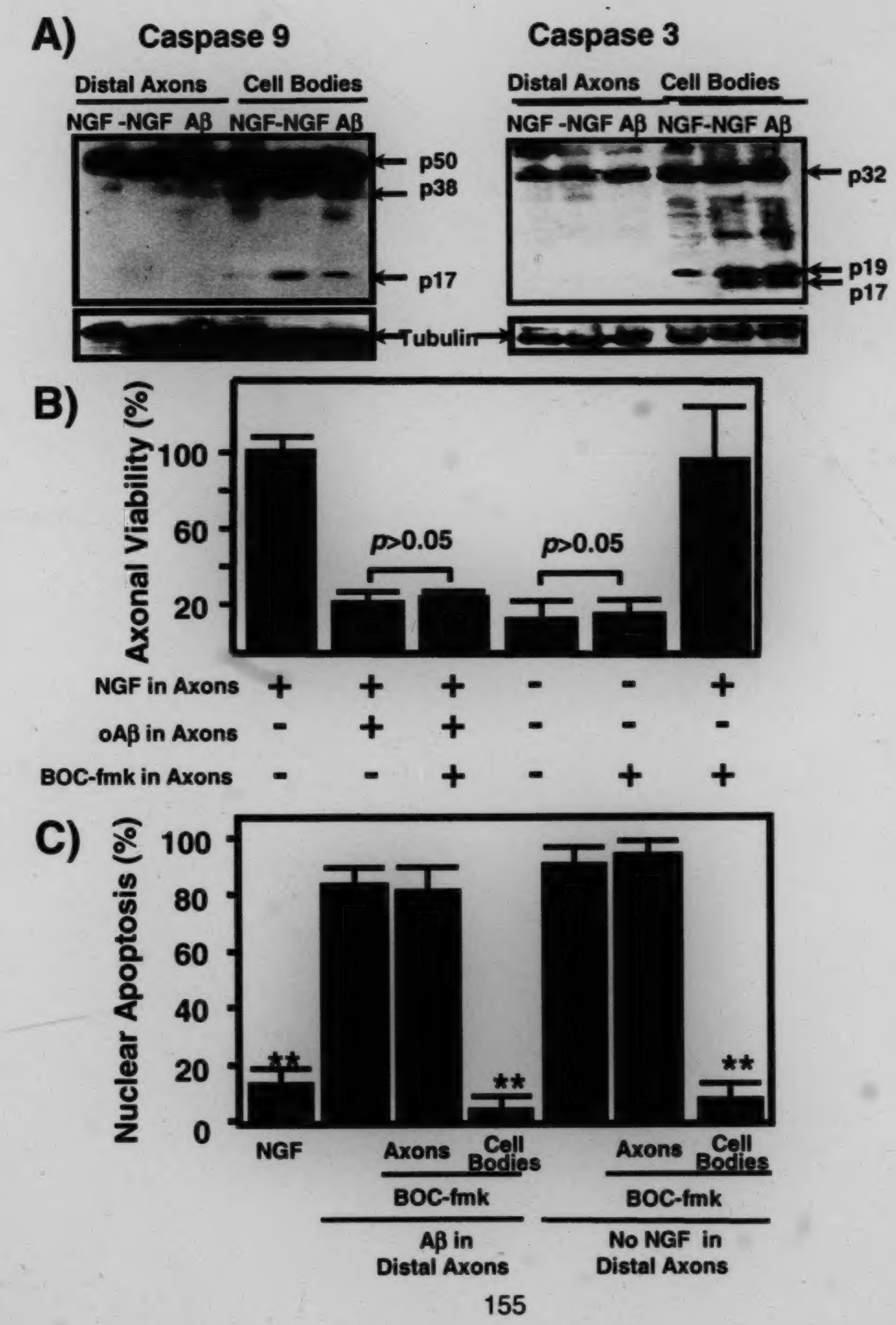
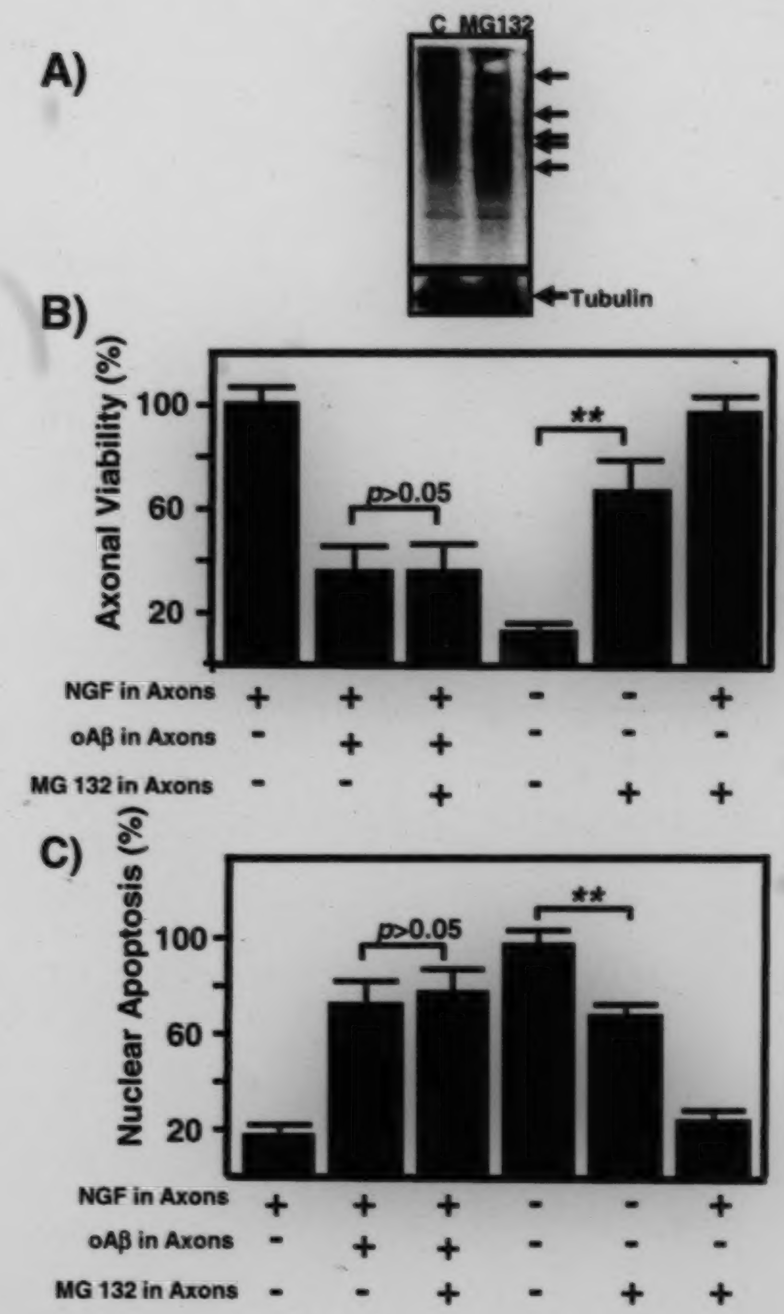

A)
Caspase 9
Distal Axons
Cell Bodies
NGF -NGF Aβ NGF -NGF Aβ
p50
p38
p17
Tubulin
Caspase 3
Distal Axons
Cell Bodies
NGF -NGF Aβ NGF -NGF Aβ
p32
p19
p17
B)
Axonal Viability (%)
100
60
20
p>0.05
p>0.05
NGF in Axons + + + - - +
oAβ in Axons - + + - - -
BOC-fmk in Axons - - + - + +
C)
Nuclear Apoptosis (%)
100
80
60
40
20
0
**
**
**
NGF
Axons
Cell Bodies
BOC-fmk
Aβ in Distal Axons
Axons
Cell Bodies
BOC-fmk
No NGF in Distal Axons

Figure 4-6. Protein degradation by the ubiquitin-proteasome system (UPS) is not required for Aβ-induced axonal degeneration. A) UPS inhibitor MG132 effectively inhibits proteasomal degradation in distal axons of sympathetic neurons. Neurons cultured in compartmented dishes were treated without or with 5 μM MG132 in distal axons for 2h. Cellular material from the distal axons-containing compartments was harvested and the presence of ubiquitinated proteins (indicated by arrows) was analyzed by immunobloting with an antibody anti-ubiquitin as indicated under Materials and Methods. **B)** Neurons cultured in compartmented dishes were treated with oAβ (20μM) in distal axons or were deprived of NGF. Some cultures were given MG132 (5μM) in distal axons together with Aβ or to NGF-deprived neurons. Axonal viability was evaluated after 36h. Values are means ± S.D. and are expressed as percentage of maximum axonal viability obtained in untreated neurons. Statistically significantly differences from cultures given no MG132 ($p<0.001$) are indicated by ** and were evaluated by the Kruskal-Wallis test with Dunn's Multiple pot hoc comparison test. **C)** Evaluation of apoptosis in cultures indicated in B). Data are expressed as means ± S.D. Statistically significantly differences from cultures given no MG132 ($p<0.001$) are indicated by ** and were evaluated by the Kruskal-Wallis test with Dunn's Multiple pot hoc comparison test. The experiment was repeated three times with similar results.


A)
C
MG132
Tubulin
B)
Axonal Viability (%)
100
60
20
p>0.05
**
NGF in Axons + + + - - +
oAβ in Axons - + + - - -
MG 132 in Axons - - + - + +
C)
Nuclear Apoptosis (%)
100
60
20
p>0.05
**
NGF in Axons + + + - - +
oAβ in Axons - + + - - -
MG 132 in Axons - - + - + +

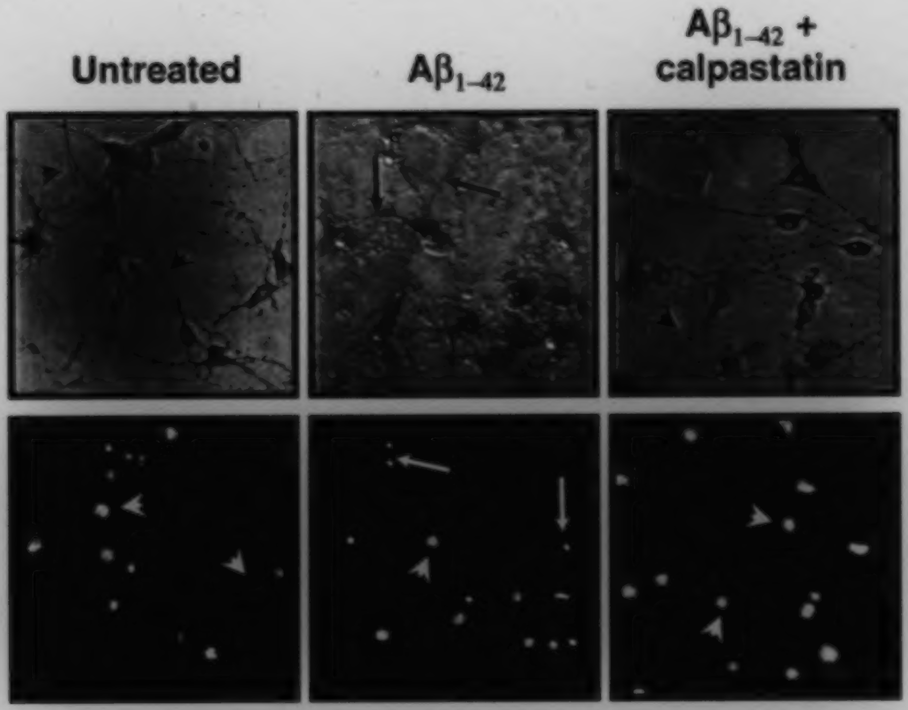


Figure 4-7. Calpastatin recovers neurite viability of Aβ-treated basal forebrain cholinergic neurons. Basal forebrain cholinergic neurons were treated with Aβ $_{1\text{-}42}$ (20 μM) without or with calpastatin (10μM). Neurons receiving calpastatin were preincubated for 4 h with the inhibitor. After 24h of treatment MTT reduction was assessed. Black arrowheads point to neurites containing the formazan compound. Black arrows indicate neurites devoid of the MTT reduction product. White arrowheads and arrows point to neuronal and apoptotic nuclei respectively.

Figure 4-8. Calpastatin protects from Aβ-induced axonal degeneration and apoptosis. Neurons cultured in compartmented dishes were treated with oAβ(20μM) or deprived of NGF. Some cultures were pre-treated for 4h with Calpastatin (10μM) in DAX and given calpastatin throughout the period of Aβ treatment. **A)** Axonal viability was evaluated by MTT reduction as described under Materials and Methods. **B)** Apoptosis was evaluated by Hoechst 33258 staining. In both cases values are means ± S.D. and are expressed as percentage of maximum viability in untreated controls **(A)**. Statistically significant differences from cultures given no calpastatin are indicated by * ($p < 0.01$) and ** ($p < 0.001$) and were evaluated by the Kruskal-Wallis test with Dunn's Multiple pot hoc comparison test. The experiment was repeated two times with similar results.

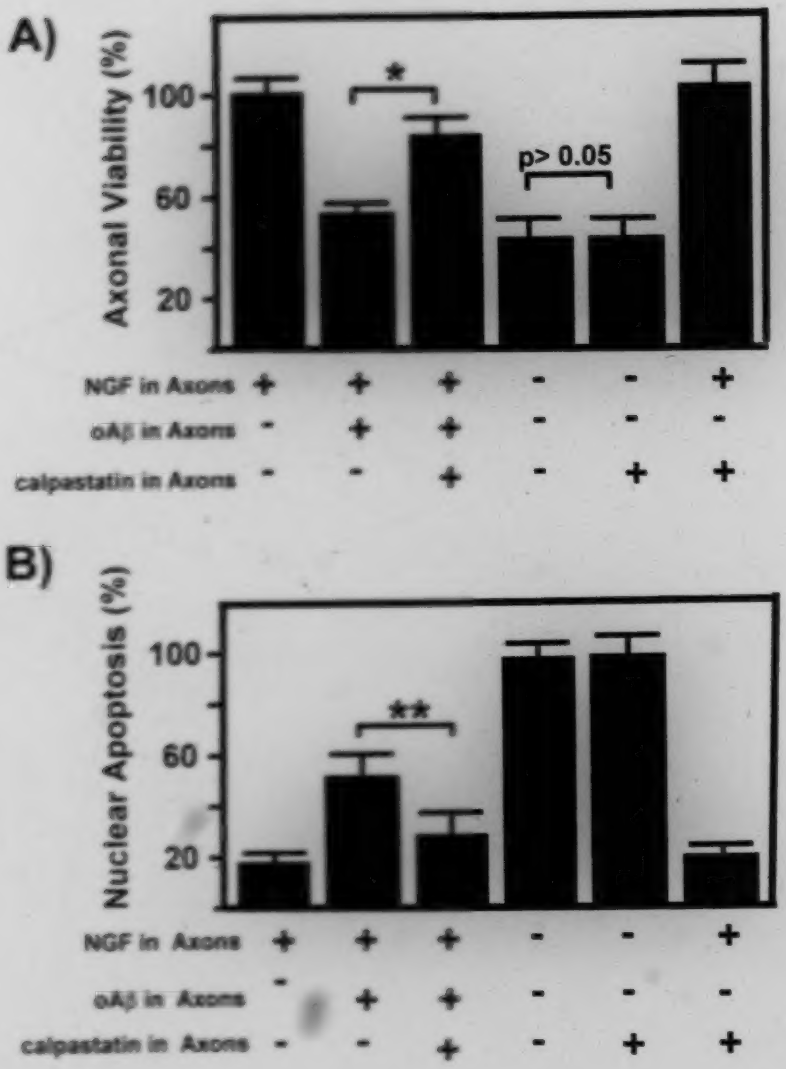
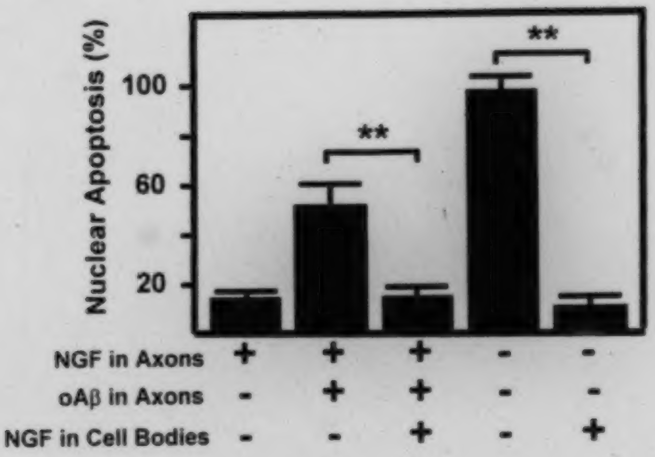

A)
Axonal Viability (%)
100
60
20
*
p> 0.05
NGF in Axons + + + - - +
oAβ in Axons - + + - - -
calpastatin in Axons - - + - + +
B)
Nuclear Apoptosis (%)
100
60
20
**
NGF in Axons + + + - - +
oAβ in Axons - + + - - -
calpastatin in Axons - - + - + +

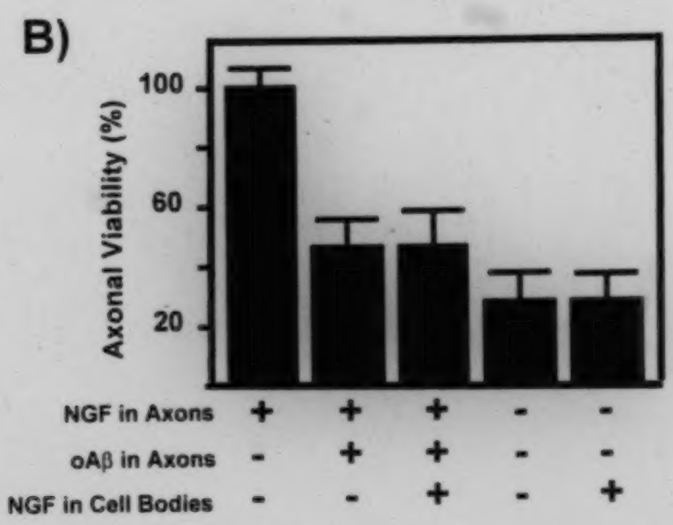
Figure 4-9. NGF provided to cell bodies protects from Aβ -induced apoptosis. Neurons cultured in 3-compartment dishes were treated with oAβ (20 μM) or deprived of NGF in distal axons. Some cultures were provided with NGF (50 ng/ml) in the cell body-containing compartment. **A)** Nuclear apoptosis was examined after 36h. Data are expressed as means ± S.D. Statistically significant differences from cultures given no NGF in CB are indicated by ** ($p<0.001$) and were evaluated by the Kruskal-Wallis test with Dunn's Multiple post hoc comparison test. **B)** Axonal viability was evaluated in the distal axons of the cultured examined in **A)**. Values are means ± S.D. and are expressed as percentage of maximum viability in neurons given NGF and no Aβ (control).

A)



Nuclear Apoptosis (%)
100
60
20
**
**
NGF in Axons + + + - -
oAβ in Axons - + + - -
NGF in Cell Bodies - - + - +

B)



Axonal Viability (%)
100
60
20
NGF in Axons + + + - -
oAβ in Axons - + + - -
NGF in Cell Bodies - - + - +

CHAPTER 5

CONCLUSIONS

The aim of this thesis was to understand the regulation of neuronal apoptosis in physiological and pathological paradigms.

In the first study, I used sympathetic neurons deprived of NGF as a model of apoptosis that occurs under physiological conditions, to examine the effect of ceramide. I demonstrated that the short chain analogue C_6-ceramide as well as endogenous ceramides protects sympathetic neurons from apoptosis caused by NGF deprivation. I also showed that C_6-ceramide is converted to endogenous long-chain ceramides. However the anti-apoptotic effect of C_6-ceramide is not entirely due to its conversion to long-chain ceramides, as C_6-ceramide itself promotes survival. In fact, activation of TrkA by C_6-ceramide represents one of the mechanisms involved in ceramide-induced survival. This study also showed that C_6-ceramide induces selective activation of PI3-kinase/Akt but not the Ras/ERK pathway. The differential regulation of signaling pathways might be explained by decreased TrkA internalization in the presence of ceramide (de Chaves et al., 2001).

A key discovery in my studies is that ceramide induces phosphorylation of TrkA in the absence of NGF. TrkA might represent a direct target of ceramide, or TrkA activation could result from alterations of the plasma membrane properties upon ceramide accumulation. Further studies are currently in progress in our laboratory investigating the hypothesis that accumulation of C_6-ceramide at the plasma membrane creates "sphingolipid-rich microdomains" at which TrkA is able to dimerize and activate in the absence of NGF (Posse de Chaves, submitted for publication).

Independent studies from our laboratory indicated that an alternative target of ceramide in the inhibition of apoptosis is serine/threonine phosphatase 1(PP1) (Plummer et al., 2005). Ceramide activates phosphatase 1 inhibiting the hyperphosphorylation of pRb that results from NGF deprivation. This study, in addition to the data presented in this thesis, indicates that multiple targets for ceramide exist in sympathetic neurons.

Another significant finding of my study is that addition of C_6-ceramide or accumulation of endogenous ceramide exclusively in distal axons is sufficient to block the apoptotic program in the nucleus, suggesting that survival signals are generated in the distal axons and transported to cell bodies in the absence of NGF. This finding is in accordance with the proposal by MacInnis and Campenot (2002) that neuronal survival can be activated by mechanisms independent of internalization and retrograde delivery of NGF to cell bodies. Several mechanisms of retrograde survival have been proposed and examined (Miller and Kaplan, 2002; Campenot and MacInnis, 2004). The model using ceramide to trigger TrkA activation and neuronal survival offers clear advantages over previous approaches since it excludes the potential delivery of minute amounts of NGF to cell bodies which might be difficult to assess.

The natural agonist that causes elevation of cellular ceramide in sympathetic neurons has not been identified. I demonstrated that selective activation of $p75^{NTR}$ by BDNF or by NGF in the presence of the TrkA kinase inhibitor K252a induces elevation of ceramide. The increase of ceramide corresponds to SM hydrolysis as demonstrated in other cell types. However, the relevance of ceramide generation mediated by $p75^{NTR}$ in sympathetic neurons is unclear since this pathway is not activated in the presence of NGF. Furthermore in the absence of NGF, $p75^{NTR}$ levels decrease dramatically and become too low to trigger the formation of sufficient ceramide. Hence, $p75^{NTR}$ does not play a role in ceramide accumulation under conditions of NGF deprivation. Further studies are required to determine the physiological/pathological relevance and the mechanism of $p75^{NTR}$ down-regulation after NGF withdrawal.

In order to understand neuronal apoptosis that occurs in pathological conditions, the second study of this thesis was directed to examine the mechanisms involved in neuronal apoptosis and axonal degeneration induced by β-amyloid peptide (Aβ) in a model applicable to basal forebrain neurons. Basal forebrain cholinergic neurons extend their axons to areas of the brain (cortex and hippocampus) in which Aβ accumulates. However, it is difficult to mimic this situation in three compartmented culture for basal forebrain cholinergic neurons, as they do not extend long axons. Therefore using a three compartmented culture system for sympathetic neurons, I mimicked the *in vivo* situation by treating neurons with Aβ exclusively in distal axons.

Although there is evidence showing that Aβ causes apoptosis as well as neurite degeneration, it is unknown if axonal degeneration is a consequence of neuronal death or conversely, it is the primary event leading to nuclear apoptosis. I demonstrated here that

$A\beta_{1-42}$ causes rapid (~6h) axonal degeneration, which is independent of neuronal apoptosis. Moreover I demonstrated that the mechanism responsible for Aβ-induced axonal degeneration does not involve local activation of caspases in axons, although activation of caspases in cell bodies takes place in response to exposure of distal axons to Aβ. Accordingly, inhibition of caspase activation in cell bodies protects from Aβ-induced apoptosis but not from Aβ-induced axonal degeneration. Inhibition of the ubiquitin-proteosome system is also unable to prevent Aβ-induced axonal degeneration but prevents axonal degeneration induced by NGF deprivation. This finding indicates that axonal degeneration caused by Aβ and NGF deprivation is activated by different mechanisms. Lastly, the calpain inhibitor, calpastatin, provided to distal axons protects from Aβ-induced axonal degeneration. Importantly, prevention of axonal degeneration by calpastatin is sufficient to reduce Aß-induced apoptosis. This finding suggests that calpains mediate Aβ-induced axonal damage and that nuclear apoptosis is secondary to axonal degeneration. Therefore, axons and calpains should be considered prime targets in the development of therapeutic strategies for Alzheimer's disease.

Several mechanisms have been proposed to mediate Aß toxic effects. They include direct interaction of Aß with cellular membranes and surface receptors (reviewed in Dickson, 2004) such as $p75^{NTR}$ (Kuner et al., 1998), RAGE (Yan et al., 1996), α7AchR (Wang et al., 2000) and amylin receptor (Jhamandas and MacTavish, 2004). Whether binding of Aß to any of these receptors activates signaling pathways that lead to calpain activation is unknown and deserves further investigation.

Based on the evidence indicating that in Alzheimer's disease the cell bodies of basal forebrain cholinergic neurons receive less NGF, it is likely that NGF retrograde transport is impaired in this disease (Salehi et al., 2004). Strategies that include the delivery of NGF to basal forebrain cholinergic neurons for treatment of Alzheimer's disease (Auld et al., 2002) are based on this concept. The study presented here demonstrated that NGF is able to protect neurons from apoptosis when given to cell bodies, supporting the notion that impairment of NGF retrograde signaling is involved in the death program activated by Aβ. Although the data indicate that axonal damage is not due to NGF deprivation, local exposure of Aß might lead to impairment of normal axonal functions such as retrograde transport.

Future investigations to determine if Aβ causes a decrease in NGF retrograde transport will help in understanding the mechanisms of basal forebrain neuron death in Alzheimer's disease.

The data presented in this thesis provide new insights into the pathogenesis of Alzheimer's disease and offer novel information for the development of effective strategies for neuroprotection.

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